Lipid-based formulations for oral delivery of poorly water-soluble drugs

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Summary

Lipid-based formulations have potential to raise the bioavailability of poorly water-soluble drugs beyond that which can be achieved from solid dosage forms. Physiological lipid processing is expected to be involved, and in this study bile salt and lecithin micelles, present in abundance during lipid digestion, were demonstrated to enhance the aqueous solubility of steroids. A trend of increased solubility enhancement with increasing steroid hydrophobicity was observed.

Lipid-based formulations frequently include surfactants with the capacity to reduce lipid digestion *in vitro*. Aspects of the ability of surfactants to reduce triglyceride digestion were investigated using an *in vitro* model, where lipase activity was monitored by titrating liberated fatty acids.

Lipase activity towards triglyceride was characterized in terms of enzyme kinetic parameters. Characterization of lipolysis in the presence of hydrophilic surfactants, with inhibitory behaviour towards lipase, revealed a lag phase which indicated that the surfactants exhibited reversible inhibition. Inhibition was thought to be overcome by colipase.

Lipase activity in the presence of a range of non-ionic surfactants suggested a relationship between the hydrophile-lipophile balance of a surfactant and the extent of inhibition. It was suggested that these observations were related to partitioning of the surfactant into the lipid interface.

Lipophilic surfactants were able to overcome lipase inhibition mediated by hydrophilic surfactants and the most effective in reversing inhibition were noted to contain lipolytic products as part of their composition. These lipolytic products were thought to clear the lipid interface, thus promoting colipase binding and lipase activity.

No conclusion could be drawn from an *in vivo* study comparing drug bioavailability from two lipid-based formulations (containing surfactants which rendered one available to lipase activity *in vitro* and the other not). This may reflect the capacity of the gut to overcome lipase inhibition due to surfactants. A mathematical model adapted for emulsions suggests a lipid-based formulation may improve bioavailability of drugs with a log P up to 5 without taking into account the additional advantages from digestion.

To my Grandma,
Vera Howlett
in her ninetieth year

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Abbreviations

(including acronyms and common symbols used).

An	absorption number
$AUC_{(0\text{-}24)}$	area under a plasma versus time curve from 0-24 h post dose
BBM	brush border membrane
bile salt	sodium taurodeoxycholate
BP	British Pharmacopoeia
C_{max}	maximum plasma concentration
CMC	critical micelle concentration
Cremophor	Cremophor RH40
cyclosporin	cyclosporin A
Dn	dissolution number
Do	dose number
ε	molar absorption coefficient
F	fraction dose absorbed
$FABP_m$	fatty acid binding protein
GI	gastrointestinal
HGL	human gastric lipase
HLB	hydrophile-lipophile balance
hPL	human pancreatic lipase
HPLC	high-performance liquid chromatography
k'	capacity factor
k_i	inhibitor constant
K_m	Michaelis constant
λ_{max}	wavelength of maximum absorption
LAEs	linear alcohol ethoxylated surfactants
n-LAE	Surfactant (e.g. LAE) with an ethoxylation number of n
LCT	long chain triglyceride
log P_{HPLC}	apparent log P determined via a HPLC method
log P_{oct}	log of the partition coefficient in an octanol / water system

lecithin	L-α-phosphatidylcholine
Marlowet	Marlowet R11
MCT	medium chain triglyceride
MMB	microscopic mass balance
Neobee	Neobee M20
Neoral	Sandimmune Neoral®
NMR	^{1}H NMR
NPEs	nonylphenol ethoxylated surfactants
ODS	octadecyl silica
PEG	polyethylene glycol
pH-stat	stationary pH device
PIT	phase inversion temperature
ppm	parts per million
PSEs	polyethoxylated sorbitan esters
RP-HPLC	reverse phase high-performance liquid chromatography
r.p.m	revolutions per minute
SCT	short chain triglyceride
SEDDS	self-emulsifying drug delivery systems
SR	solubilization ratio
S/S_o	solubility enhancement ratio (solubility in micellar solution as a fraction of the intrinsic solubility in water)
TBU	tributyrin unit
TGME	tetraethylene glycol monooctyl ether
t_{max}	time to reach maximum plasma concentration
$t_{\%}$	time taken for pancreatin to convert a specified percentage of triglyceride to monoglyceride and two fatty acids
t_R	retention time
USP	United States Pharmacopoeia
UWL	unstirred water layer
VLDL	very low density lipoproteins
V_{max}	maximum reaction velocity
$V_{\%}$	percentage of uninhibited initial velocity

Chapter 1 - The benefit of lipid-based formulations in relation to the bioavailability of poorly-water soluble drugs.

1.1 General Introduction

Prolonged dissolution is frequently held responsible for poor bioavailability of hydrophobic drugs when drug is presented in a crystalline state within the dosage form. Administration of a hydrophobic drug dissolved in a lipidic solvent could be presumed to avoid the dissolution stage hence bioavailability may be improved and/or a more consistent profile of drug absorption with time observed.

The ability of lipid-based formulations to improve absorption of poorly-water soluble drugs when compared to the use of aqueous suspensions or solid dosage forms has been subject to wide discussion[1] as have the possible mechanisms involved. Early indications that the bioavailability of a poorly-water soluble drug may be improved due to the presence of lipid were gleaned from observations of increased drug absorption upon dosage with a high fat meal[2, 3].

Presence of food in the gastrointestinal (GI) tract may alter the bioavailability of a particular drug through various mechanisms as reviewed by Charman[4].

Physicochemical interactions between food and drug and the resultant effect upon bioavailability will depend on individual characteristics of the drug, the nature of the formulation, the interval between drug and food ingestion and the nature of the food eaten[5]. In addition alteration in enzymatic activity and the environment of the GI tract upon conversion from the fasted to the fed state are of prime consideration with regards to drug bioavailability. The effect of fatty food is of major interest in this context due to the scope for improvement in the dissolution rate of a poorly-water soluble drug through stimulation of the lipid digestion cascade[4].

Activation of lipid digestive processes may occur via administration of drug with a dietary source of lipid or by presentation of the drug within a lipid-based formulation. The use of a simple solution of drug in lipid is rarely possible due to the limited solubility of some hydrophobic drugs in lipids. This generally leads to the design of more complex lipid-based formulations containing other components such as co-solvents and surfactants. The nature of these components requires further

consideration as they may also influence the lipid digestion cascade removing any advantage to drug bioavailability conferred by the use of lipid in a formulation. A brief review of the stages of dietary lipid digestion is given in section 1.2 followed by a discussion of their possible influence upon the bioavailability of hydrophobic drugs (section 1.3). Lipid formulations are briefly described (section 1.4) followed by an overview of the work presented in this thesis.

1.2 The lipid digestion cascade.

Over 97 % of dietary lipid is ingested in the form of triglycerides,[6] the majority of which are long chain (chain length C_{16}-C_{18})[7]. The lipid digestion cascade describes the three steps involved in the conversion of triglyceride into a useful energy source[8]. Briefly lipid assimilation commences with emulsification of the triglyceride into a coarse dispersion of droplets. These provide a high surface area for enzymatic hydrolysis of triglyceride into monoglycerides and fatty acids. These lipolytic digestion products are absorbed by the intestinal enterocytes and subsequently transported into the portal vein or the lymph. The chain length of the triglyceride determines the route taken at certain stages of the cascade.

1.2.1 Lipids within the stomach.

Lipid emulsification occurs in the stomach where the process is augmented by the hydrolytic activity of human gastric lipase (HGL). HGL has been located inside granules in the apical region of chief cells[9] and is mainly active in the fundic portion of the stomach[10]. The enzyme is able to hydrolyze short, medium and long chain triglycerides resulting in the release of diglycerides and fatty acids[11]. The velocity of HGL activity is dependent upon chain length of the triglyceride[12] and pH, with optimal activity shown at a pH value of 5.4[13]. In addition the enzyme has been shown to exhibit partial selectivity for the *sn*-3 ester bond[13]. Overall HGL has been proposed to be responsible for between 10 to 30 % of total gastrointestinal lipolytic activity[8]. *In vitro* studies have demonstrated a correlation between HGL activity and interfacial tension at a triglyceride / water interface[14, 15]. Inclusion of amphiphiles such as bile

salts and some proteins promoted HGL activity by reducing interfacial tension whereas the higher pressure of a pure triglyceride / water interface was noted to inhibit HGL. This was suggested to be a result of protein unfolding due to vulnerability of the enzyme structure to denaturation.

Triglyceride and ingested lecithin in normal dietary proportions (between 50:1 to 20:1) tend to form an emulsion system consisting of triglyceride droplets surrounded by an outer close packed monolayer of lecithin[8]. The lecithin monolayer decreases interfacial tension, thus helping to stabilize the emulsion by preventing droplet coalescence and increasing the activity of HGL. Partially digested proteins, complex polysaccharides and membrane derived phospholipids also present in the stomach possess emulsification properties and can decrease surface tension further thus promoting HGL activity[8]. Diglycerides and fatty acids resulting from HGL activity may also further emulsification by lowering surface tension of the triglyceride droplets[13]. The presence of these lipolytic products has the added advantage of promoting immediate pancreatic lipase activity upon release of the emulsion into the duodenum[16].

Another important factor in emulsification is thought to be mechanical forces of the stomach antrum[17] where contractions increase intraluminal pressure. The stomach contents are pushed towards the closed pylorus and then retropelled back to the stomach body providing mixing followed by applied shear forces tearing the liquid interfaces apart when chyme is released through the pylorus.

Any short and medium chain fatty acids released via HGL activity will be absorbed through the stomach mucosa due to their aqueous solubility in unionized and ionized forms. The long chain fatty acids are unionized at stomach pH and partition mainly into the core of the lipid droplet in a liquid state ready for transference to the duodenum[8].

1.2.2 Duodenal digestion - the role of pancreatic lipase and colipase.

The enzyme responsible for the majority of triglyceride hydrolysis in the duodenum is pancreatic lipase although some HGL activity will remain whilst the pH of chyme is between 5 and 7[18]. Pancreatic lipase activity is minimal unless the substrate is present

in sufficient concentration to form an interface, a phenomenon known as interfacial activation[19]. The presence of amphiphiles such as bile salts, phospholipids and proteins at the surface of the emulsion droplets prevents binding of pancreatic lipase to the triglyceride interface. Under these conditions binding of pancreatic lipase is only possible in conjunction with colipase, a non-enzymatic protein cofactor[20].
Binding affinity of colipase and thus pancreatic lipase to the lipid interface is enhanced by long chain fatty acids already present in the core of the emulsion droplets from previous HGL activity. These rise to the droplet surface in the higher pH environment of the duodenum and mix with dietary phospholipids to enhance colipase binding[21].
Upon anchorage at the triglyceride interface the hydrolytic activity of pancreatic lipase releases lipolytic products of *sn*-2 monoglycerides and fatty acids[22]. Diglycerides located at the lipid interface from previous HGL activity are also readily available for further hydrolysis by pancreatic lipase.

Initially lipase is secreted together with colipase in a proform (procolipase) from acinar cells in the pancreas. Procolipase is activated into colipase by trypsin cleavage[23] resulting in removal of the N-terminal pentapeptide which has been proposed to play a role in the regulation of fat intake[24]. The purpose of procolipase cleavage is unclear as both procolipase and colipase are capable of promoting lipase mediated hydrolysis of triglyceride at the lipid interface in the presence of bile salts and phospholipid[25]. However under certain reaction conditions colipase was found to be more efficient at lipase activation than procolipase.
Insight into binding and activation of human pancreatic lipase (hPL) at a lipid interface has been achieved via crystallographic techniques. Resolution of the 3D-structure of hPL via X-ray crystallography revealed hPL to be a single chain glycoprotein of 449 amino acids arranged as two domains connected by a hinge region[26]. Residues 1-336 form the amino terminal domain[26] typical of an α/β hydrolase fold[27] with a dominant central parallel β sheet. Residues 337-449 form a carboxy terminal domain which subsequent studies of hPL crystallized with procolipase demonstrated to contain a binding site for a 1:1 stoichiometric complex of enzyme with cofactor[28]. In addition procolipase was observed to consist of three finger shaped regions with a significant

proportion of non-polar amino acids concentrated in the finger tips; these were proposed to provide a means of binding procolipase to the lipid interface[28, 29].

The amino terminal domain contains the active site which is a catalytic triad of the amino acid residues, Ser 152, Asp 176 and His 263[26] with Ser 152 being the nucleophilic residue essential for catalysis[30]. The active site is covered by an amphipathic helix[31] acting as a lid therefore conformational change of the enzyme is necessary to allow substrate contact with the active site. Rolling back of the lid uncovers the hydrophobic catalytic center and augments the hydrophobicity of the surface surrounding the active site by exposure of the nonpolar side of the lid[31].

The 3-D structure of a hPL-procolipase complex, co-crystallized with mixed micelles of phosphatidylcholine and bile salt revealed further conformational changes in the enzyme-cofactor complex[32]. With phospholipid bound in the active site the position of the catalytic triad is unchanged although the surrounding environment is altered. Van der Waals bonds exist between a surface loop (β5 loop) present over the active site and the lid when the enzyme is in the closed conformation. When the lid rolls back the β5 loop folds onto the core of the protein rendering the active site serine accessible to solvent in the base of an hydrophobic canyon ideal for lipid substrate binding.

The same study[32] illustrated that repositioning of the lid and β5 loop in hPL also results in creation of an electrophilic region (oxyanion hole) around the active site which acts to stabilize the negative charge generated during ester hydrolysis of the substrate. Further interaction also occurs between the N-terminal region of procolipase and the open conformation of the lipase lid via direct H-bonds giving increased stability to the hydrophobic surface around the active site in preparation for interaction with lipid interfaces. Chemical modification of a procolipase residue involved in these H-bonds resulted in loss of procolipase activity illustrating the importance of lid binding for functioning of the complex[24]. Deletions in the lid domain of hPL have given further support for involvement of the lid in interfacial binding and activation[33]. The bending angle between the C and N terminal domains of hPL has also been suggested to be important for positioning of the lid and subsequent catalytic activity[34].

Crystallization of the hPL-colipase complex with an inhibitor of lipase, a C_{11} alkyl phosphate compound synthesized to mimic a triglyceride substrate, revealed

information regarding the possible interactions between substrate and enzyme[35]. The long hydrophobic C_{11} alkyl chain, taken to represent a fatty acid chain of a triglyceride molecule, was orientated in the hydrophobic groove of the active site with van der Waals contacts formed with the active site Ser 152. Interaction of the C_{11} alkyl chain was also observed with hydrophobic areas on the open lid near the rim of the active site. Assuming these results to be analogous to binding of a triglyceride molecule the remaining fatty acid chain was suggested to be positioned within the lipid layer thus enabling rapid removal of the lipolytic products.

The lid movement and consequent reorganization of pancreatic lipase upon lipid binding appears to have many functions, an interfacial lipid binding site is created, shape and access is given to the active site canyon, the oxyanion hole is stabilized and interaction with procolipase is strengthened[32]. The stimuli for opening the hPL lid is unknown[35] and may simply occur in the presence of a lipid interface. Alternately hPL may exist in dynamic equilibrium between a closed and open form, the open conformation of which is stabilized in that conformation by contact with a lipid interface.

The sequence of events leading to the formation of the lipase-colipase-lipid complex is also unclear with possible binding of lipase with colipase in solution before adherence to the interface or via initial binding of colipase to the lipid interface acting as an anchor for subsequent lipase attachment. Both pathways have been postulated to exist by Lowe[36] who suggested binding between colipase and the N-terminal of hPL to be a crucial factor in potentiating lipase activity. Further consideration of experimental evidence suggests that although binding of lipase and colipase is possible in solution, preliminary binding of colipase with the lipid interface may induce a conformational change increasing affinity of colipase for lipase[37].

1.2.3 The physicochemical state of lipids during digestion.

The physicochemical state of aqueous upper intestinal contents is of importance in understanding the transport of lipolytic products from the emulsion surfaces to the enterocytes and in the context of the work here transport of drug molecules by the same means. Lipid emulsification mechanisms of the stomach have been suggested

from results of an *in vivo* study to produce partially emulsified lipid droplets of between 1 to 100 μm diameter with the largest fraction sized in the range of 20 to 40 μm[17]. An extension of the investigation revealed no further marked emulsification of dietary fat in the duodenum compared to the stomach which was proposed to be a result of limited mechanical shear forces despite the presence of excess amphiphiles[38]. Most dietary lipids were present in the human duodenum as emulsified droplets 1 to 50 μm in size after 1 hour of digestion. Lipid droplets of the same size in the stomach and duodenum differed in lipid composition with the higher lipolytic activity in the duodenum reflected by neutral lipid depletion and duodenal droplet enrichment with lipolytic products, cholesterol and phospholipids[38].

Accumulation of lipolytic products at the emulsion surface appears to be the first step in creation of the two separate phases suggested to exist (from *in vitro*[39] and *in vivo* investigations[40]) together with emulsion droplets in postprandial duodenal contents. A model to explain the lipid phase states present throughout lipolysis[40] is described below. Duodenal absorption of long chain fatty acids previously released from triglyceride by HGL activity stimulates ejection of pancreatic enzyme secretion[8]. Biliary lipids (bile salt and lecithin mixed micelles and cholesterol) are also released as bile from the gallbladder and rapidly become diluted in chyme from the stomach. Further rapid interfacial hydrolysis forms a multilamellar liquid crystalline bilayer at the surface of the emulsified lipid droplet which is comprised mainly of monoglycerides and fatty acids, with phospholipid, cholesterol and bile salts also present. Continued lipolytic activity shrinks the neutral lipid core of the droplet with the resultant high surface pressure releasing the liquid crystal bilayer as a multilamellar vesicle.

Layers from the multilamellar vesicles bud off and become solubilized by unsaturated bile salt micelles into unilamellar vesicles with hydrodynamic radii of between 400 to 1200 Å. The unilamellar vesicles are probably the initial phase in the process of lipolytic product dispersal, being comprised of aqueous mixed lipids saturated with bile salts. The structure of lipid aggregates in the small intestine thus depends on the mixed lipid to bile salt ratio as upon further bile salt incorporation into the unilamellar vesicles smaller intestinal mixed micelles are formed (hydrodynamic radii of 80 Å). The intestinal mixed micelles contain bile salts, long chain fatty acids, monoglycerides,

cholesterol and phospholipids and act as a sink for lipolytic products under normal *in vivo* conditions.

Solubilization of lipolytic products (formed by the action of pancreatic lipase, phospholipase and cholesterol esterase) within intestinal mixed micelles increases their luminal solubility in addition to providing a means of diffusion to their site of absorption. Micelle formation is not required for absorption of short chain fatty acids as their high solubility in aqueous chyme[41] enables these molecules to freely diffuse to the brush border membrane (BBM), although their hydrophilic nature does provide some resistance to their subsequent passage across[42].

1.2.4 Lipid absorption.

The absorption of monoglycerides, medium and long chain fatty acids through the BBM of the intestinal mucosa occurs mainly from intestinal mixed micelles, although evidence suggests a certain amount of absorption is also possible from the unilamellar vesicular phase[43]. Absorption usually occurs by a process of passive diffusion due to the lipophilic nature of the products. However potential barriers to the passage of lipolytic products exist next to the BBM membrane[42], namely the unstirred water layer (UWL) and intestinal mucus.

Dissociation of the lipolytic products from the vesicles and micelles is necessary before absorption[44]. Three models (reviewed by Thomson[45]) have been proposed to explain lipolytic product transfer from the micellar phase to the BBM. The collision model describes partitioning of the fatty acid directly from the micelle into the BBM of the enterocyte[42]. The other models involve a further step whereby fatty acids first partition into an intermediate aqueous phase. This is the UWL where fatty acids are soluble enough to diffuse across for absorption by passive transport, i.e. the "aqueous" model[45].

Within the UWL acid microclimates are present adjacent to the BBM[46]. These have been suggested to be formed by mucus entrapment of hydrogen ions transported into the intestinal lumen by various types of antiporters[47, 48]. If the intermediate aqueous phase is an acid microclimate, fatty acid uptake may occur via the "dissociation" model[45]. The decreased pH of the environment ensures fatty acids are unionized,

decreasing their solubility in micelles and enhancing partitioning into the BBM[49]. In addition low pH increases the critical micelle concentration (CMC) leading to further dissociation of micelles and fatty acid release[50].

Although transport of fatty acids across the BBM into enterocytes has been thought to occur via passive diffusion[51], evidence suggests absorption may be enhanced by the presence of a BBM fatty acid binding protein ($FABP_m$)[52]. $FABP_m$ has been located on the outer surface of the enterocyte membrane and is thought to act as a medium and long chain fatty acid receptor[53], possibly aiding lipid transport into the cell by acting as a translocase[54].

Upon dissociation of the intestinal mixed micelles, the bile salt molecules diffuse back to the intestinal lumen to form new micelles which take up further lipolytic products until lipid absorption is complete. Some bile salts are then passively absorbed in the jejunum with the majority undergoing active resorption in the terminal ileum[55] for transport to the liver via the enterohepatic circulation.

1.2.5 Cellular events and lymphatic transport.

Once inside the enterocyte long chain fatty acids and monoglycerides are transported through the cell cytoplasm to the endoplasmic reticulum. The mechanism for transportation is still unknown however intracellular fatty acid-binding proteins detected in enterocytes may be involved[56]. Reesterification of the lipolytic products into triglyceride then occurs via two possible pathways (reviewed by Tso[7]); the extent to which each pathway is utilized depending upon the lipid mixture absorbed. The monoglyceride pathway involves monoglyceride and free fatty acid together with the enzyme complex, triglyceride synthase, situated at the cytoplasmic surface of the endoplasmic reticulum[57]. When monoglyceride supply is low the α-glycerophosphate pathway converts absorbed fatty acids and endogenous glycerol into triglyceride within microsomes[45].

The re-esterified triglyceride is utilized in the endomembrane system[58] as the source of core lipid for the assembly of lipoproteins[45], namely chylomicrons and very low density lipoproteins (VLDL). These are spherical complexes consisting of a triglyceride core surrounded by a coat of cholesterol, phospholipid and apoproteins.

Chylomicrons and VLDL stabilize lipid in the aqueous media of the cytoplasm and lower cellular concentration of lipid to maintain a concentration gradient for passive absorption. Eventually they migrate towards the basolateral membrane and are released by exocytosis into the intercellular space[59]. Passage of chylomicrons and VLDL through the lamina propria results in accumulation in the mesenteric lymph for final secretion via the thoracic duct into the systemic circulation[60].
In the case of short and medium chain fatty acids (less than 12 carbon atoms) these in general do not undergo re-esterification into triglyceride and are passively absorbed along with glycerol directly into the portal blood[61].

1.3 Influence of the lipid digestion cascade on drug absorption from lipid-based formulations.

The physiological stages of lipid processing may have a beneficial effect upon the absorption of a poorly-water soluble drug if activated by co-ingested lipid or lipid included in the formulation. Possible mechanisms by which stimulation of the lipid digestion cascade may improve drug bioavailability are discussed below:

1.3.1 Gastric emptying.

Chyme formed from a fatty meal contains some lipolytic products released by HGL activity, which upon entry into the duodenum, have been demonstrated to reduce the rate of gastric emptying[62]. This effect is possibly mediated by enhanced release of cholecystokinin, a polypeptide hormone known to be involved in slowing gastric emptying[63]. Physiologically a decreased rate of gastric emptying is advantageous to maximize energy obtained from ingested triglyceride, as rapid gastric emptying of fat has been demonstrated to diminish the efficiency of lipolysis[64].
In terms of drug bioavailability the possible benefit of a reduction in gastric emptying rate is immediately evident, as increased gastric retention would prolong the drug absorption phase. Early work[65] reported a twofold increase in bioavailability of micronized griseofulvin when administered to humans in a corn oil in water emulsion over that obtained from an aqueous suspension and a tablet dosage form; comparable

experiments using rats[66, 67] also demonstrated improved bioavailability when drug was presented in a corn oil in water emulsion. The improved bioavailability was suggested to be due to fatty acids released from digestion of the corn oil acting to inhibit gastric emptying and thus decrease the rate of transfer of drug into the intestine[65]. Drug therefore would have more time to dissolve in the region of the intestine where absorption is likely to be most efficient.

1.3.2 Emulsification

The emulsification of a lipid formulation could be presumed to be beneficial due to the larger surface area available for drug release and enzymatic activity. Emulsification of a lipid formulation may be achieved by the normal physiological emulsification process already described, by dosage as a prepared emulsion or by inclusion of surfactants.

The classic studies using griseofulvin[66] and phenytoin[68] compared drug bioavailability from a corn oil in water emulsion, a corn oil suspension and an aqueous suspension. Control experiments demonstrated that differences achieved in drug absorption could not be attributed to solubility of the drugs at the time of dosing. For both drugs bioavailability was highest from the prepared emulsion, although in the case of phenytoin both lipid formulations gave higher bioavailability than the aqueous suspension.

Comparison of these lipid formulations in terms of their initial emulsification status is complicated by the availability of corn oil to lipase activity. The action of lipase may result in accumulation of lipolytic products upon the oil surface which could promote further emulsification and a consequent reduction in droplet size of both corn oil based formulations. The increased drug plasma levels from the prepared emulsion compared to those from the corn oil suspension nevertheless may be due to a difference in droplet size. The release of drug from the corn oil suspension could be assumed to be delayed as digestion is necessary to reduce droplet size to that equivalent of the prepared emulsion.

When phenytoin was administered to rats in a sesame oil suspension and emulsion[69] the bioavailability and maximum plasma concentrations were not significantly different

between the two formulations, although the time to reach maximum plasma concentration (C_{max}), was twice as long for the oil suspension. Compared to the aqueous suspension included in the study both oil based formulations gave an approximate 50 % increase in C_{max}. This perhaps illustrates that although use of a crude prepared emulsion can increase absorption rate by avoiding the immediate need for emulsification, if sufficient time is available for digestion and absorption a simple suspension of drug in lipid can be equally effective as an emulsified formulation to enhance bioavailability for some drugs.

Inclusion of surfactants in a lipid formulation can further reduce droplet size, with creation of a fine dispersion upon release of the formulation into gastric fluid. The features, formulation and advantages of these systems, described as self emulsifying drug delivery systems (SEDDS), are discussed further in Chapter 5.

1.3.3 Digestion

If the lipid digestion cascade is to improve bioavailability of a drug, the lipid used in the formulation must be a suitable substrate for lipolytic enzymes. The advantage of digestible lipid has already been discussed with regards to emulsification mediated by lipolytic products. In addition lipolytic products would also have a stimulatory effect on gallbladder evacuation, causing bile release with a resultant increase in the concentration of bile salts and lecithin present. The surfactant properties of bile salts and lecithin may provide a further means of enhancing drug bioavailability by increasing the solubility of a drug through micellar solubilization; drug dissolution rate may also be increased due to a reduction in surface tension. Enhancement of the solubility and dissolution rate of hydrophobic drugs by bile salt and lecithin micelles and the possible mechanisms involved are addressed in Chapter 6.

Drug bioavailability has previously been reported to be higher from formulations containing a digestible versus a non-digestible lipid[70, 71]. The highly lipophilic compound, probucol, was presented to rats as a solution in long or medium chain triglyceride (MCT) and in a non-digestible lipid (liquid paraffin)[72]. The absorption of probucol was demonstrated to be dependent on the lipid vehicle, with no drug absorption detected when using liquid paraffin. This indicated that digestion was

required to transfer drug into the GI fluid and possibly micelles from which absorption was possible, rather than drug remaining partitioned in a non-digestible lipid until excretion.

Absorption of the poorly water-soluble drug SL-512 in rats was investigated[73] using a digestible MCT formulation and a synthetic non-digestible lipid (MBLA). SL-512 exhibited the same partitioning behaviour from both lipid vehicles into an aqueous solution however serum levels were 4-fold higher from the MCT formulation. Subsequent control experiments in the same study demonstrated the importance of lipid digestion to increase absorption of SL-512, possibly explained by digestion enhancing release of drug partitioned in the lipid.

Digestion of lipid in a formulation does not necessarily need to be rapid to improve drug absorption by the mechanisms described above. The rate and extent of lipid digestion is known to differ depending on the length and degree of saturation of the fatty acid chain, as well as the extent of esterification of the glycerol backbone[1]. Choice of a lipid susceptible to rapid lipolysis may in fact be disadvantageous as absorption of lipolytic products could occur before the drug has been solubilized into mixed micelles, leading to precipitation of drug in the GI fluid.

This has been demonstrated to a certain degree by examination of the bioavailability of penclomedine[74], a poorly water-soluble cytotoxic agent, after intraduodenal administration to rats in various oil in water emulsions. Results showed an increase in bioavailability according to the lipid used in the formulation in the following order: MCT > long chain triglyceride (LCT) > non-digestible oil > short chain triglyceride (SCT) > aqueous suspension. Low bioavailability from the emulsion formed using the SCT, tributyrin, was proposed to be due to the rapid hydrolysis of tributyrin into water soluble products. The resulting lack of intestinal mixed micelles to solubilize drug and the rapid manner of drug release probably caused the drug to precipitate, hence little improvement in bioavailability was observed over than given by the aqueous suspension. Lower bioavailability of penclomedine from the LCT formulations compared to MCT formulations may be due to the slower rate of hydrolysis of LCT, with the main factor responsible for bioavailability from the LCT formulations being the ability of penclomedine to partition from the oil. The surprisingly high bioavailability of drug from the non-digestible formulation was also

suggested to be due to the same mechanism and illustrates the advantage of a dispersed formulation to increase drug partitioning into the aqueous GI fluid.
The advantage of using a digestible lipid for a formulation appears related to the physicochemical nature of the drug; a drug with a high partition coefficient would remain in the oil and therefore be more dependent upon digestion to enhance absorption. For a drug with a higher tendency to partition into the aqueous environment, simply presenting the drug in a readily dissolved state in a lipid-formulation, with good dispersion characteristics, may be sufficient to achieve higher bioavailability over that possible from a solid dosage form.
A further point for consideration is that a drug which does become solubilized through digestion will probably be partitioned within all phases created during the process, namely emulsion droplets, multilamellar and unilamellar vesicles and intestinal mixed micelles[4]. As digestion proceeds with time the relative proportions of the various phases will change accordingly, thus the absorption rate of drug from the final phases of lipid digestion may also be dependent upon the kinetics of the digestive process. This may perhaps explain why a longer period of time has been reported for drug to reach maximum plasma levels from a lipid-based formulation compared to an aqueous suspension, although eventually the lipid formulation has higher bioavailability.

1.3.4 Absorption

The presence of lipolytic products may influence the extent of hydrophobic drug absorption from the intestine in terms of drug transfer to the BBM and alteration in membrane permeability to the drug molecules[75]. The models already discussed for transfer of lipolytic products from the micellar phase to the BBM (section 1.2.4) may also apply to drug molecules sufficiently lipophilic to be solubilized within intestinal mixed micelles. These micelles are believed to solubilize high concentrations of hydrophobic drug and facilitate drug diffusion across the UWL to the BBM[70]. Upon breakdown of the micelle dissolved drug monomers are released in high concentration into the aqueous fluid surrounding the microvilli, ready for absorption across the BBM and subsequent transport into the bloodstream[70]. Micellar dissociation leading

to the release of drug in a readily absorbable form has been suggested to be promoted by absorption of bile salts in the distal portion of the small intestine[76].

The UWL, with possible augmentation from the integral mucus layer has been suggested to be the main barrier to absorption of lipophilic drugs[77-79] with restriction from the UWL reported to increase with drug hydrophobicity[1]. Recent work has demonstrated the ability of a single lysophospholipid molecule to seed aggregation of bile salt molecules at concentrations below their CMC[80]. The seeding of these aggregates appeared to be a non-specific hydrophobic mechanism therefore a suitable hydrophobic drug molecule may also be able to induce the same effect. The drug molecule would thus become surrounded by a small aggregation of bile salts with a subsequent increased transfer rate through the UWL. This proposed mechanism may perhaps play a role in hydrophobic drug absorption observed when bile salt concentration and lipolytic activity is low.

Although micellar solubilization may improve drug diffusion through the UWL, the subsequent release of lipolytic products upon dissociation of the micelle has been suggested to be disadvantageous to drug absorption. Contrary to increasing lipophilic drug bioavailability, the presence of fatty acid has been noted to restrict the absorption of griseofulvin; an effect which was explained by the ability of fatty acids to increase the thickness of the UWL and reduce intestinal blood flow[81]. Likewise extensive solubilization of drug in micelles may not always be beneficial in terms of bioavailability. Micellar solubilization of drug may result in a reduction in the rate of diffusivity compared to free drug molecules[82]. Retention of drug within micellar structures may also reduce the concentration of free drug available for absorption[79, 83].

The normal process of lipid digestion and absorption has been suggested to impair the integrity of the intestinal epithelium on a regular basis, with the extent of damage increasing with fatty acid concentration[84]. Constituents of intestinal mixed micelles and other components likely to be present during digestion of a lipid-based formulation, i.e. fatty acids, bile salts, monoglycerides, lysolecithin and non-ionic surfactants have also been reported to increase membrane permeability[85]. In the case of lipolytic products this effect may be mediated by incorporation into the membrane, altering the configuration in such a manner that it becomes easier for drugs to permeate[75]. Enhanced membrane permeability due to lipid digestion is unlikely to

increase the bioavailability of hydrophobic drugs as transport across the membrane generally occurs by passive transcellular diffusion[86]. However the absorption of highly polar and / or high molecular weight drugs may be improved[75].

Poelma et al[79] examined the influence of mixed bile salt, lecithin and fatty acid micelles upon drug absorption in terms of rate of drug transfer across the UWL, (including integral mucus layer) and epithelial membrane. The absorption of a range of hydrophilic and lipophilic drugs, solubilized within the micellar solutions was assessed using a rat chronically isolated small intestinal loop. Any influence from the micellar systems on dissolution rate of the drug was thus eliminated. Micellar concentrations were selected to be physiologically representative. Overall results from the study indicated that mixed micelles did not have any beneficial effect upon transference of solubilized drug across the UWL or the epithelial membrane for all drugs tested. Lack of absorption enhancement for the hydrophilic drugs may perhaps be related to the low concentration of oleic acid used in this study (5 mM). To demonstrate membrane permeability Kvietys et al[84] used higher oleic acid concentrations (10-40 mM) which they stated as typical of levels in the physiological fed state. The concentration of fatty acid present solely from digestion of a lipid-based formulation may not be sufficient to exert any influence by the mechanisms described above.

1.3.5 Lymphatic absorption of drugs.

The primary route of drug transport from the enterocyte into the systemic circulation is the portal vein although entry is also possible via the intestinal lymph. One of the functions of the lymphatic system is to drain fluid from extracellular tissue back into the vascular compartment. Within the GI tract the lymphatic system also provides a means of transport to the systemic circulation for lipophilic digestive products unable to disperse easily in the aqueous environment of blood, such as cholesterol[87] and fat soluble vitamins[88]. Some highly lipophilic drugs also gain access to the systemic circulation via the lymphatics (as reviewed by Porter and Charman[60]), however for a drug to be transported by this route concurrent synthesis of the triglyceride core of chylomicrons is necessary. The triglyceride for chylomicron formation is synthesized from long chain fatty acids absorbed during normal lipid digestion[89] therefore

ingestion of lipid followed by activation of the lipid digestion cascade could be presumed to enhance drug absorption via this route[90].

Selective absorption into the lymph has been demonstrated for the pesticide DDT[91] and the drug probucol[72], with evidence that co-administration of these compounds with lipid can enhance lymphatic absorption[72, 92]. The extent of absorption was found to depend on the type of lipid vehicle, with LCT revealed to give the highest concentration of drug in the lymph when compared with administration in MCT and liquid paraffin. LCT was also found to be the most effective vehicle for increasing lymphatic transport of the retinoid, isotretinoin[93]. By selection of a LCT the subsequent absorption of long chain fatty acids into the enterocyte was postulated to stimulate the synthesis of chylomicrons and hence increase capacity of the lymph to transport lipophilic drug molecules. However limited drug uptake into the lymphatic system is also possible without lipid presence[94] if drug is presented in a sufficiently solubilized form to enable drug absorption into the enterocyte. Absorption is then followed by transportation to the lymph in association with VLDL produced from endogenous lipids.

As the portal vein has a much higher flow rate than the lymph a selective uptake mechanism is required for lymphatic transport of drug to occur[95]. Methods to enhance drug absorption by the lymphatics have been widely investigated as this route has the advantages[96] of avoidance of first pass metabolism, improved absorption of highly lipophilic drugs and sustained release to the systemic circulation. In addition drug targeting strategies to areas supplied by the lymph could be useful, such as cytotoxic drugs to prevent lymph metastasis and drug treatment of immune diseases.

Drug lipophilicity appears to be a major factor with regards to whether drug will be transported via the lymphatics and attempts have been made to promote lymphatic drug transport by using prodrugs with increased lipophilicity[60]. This can be brought about by forming an ester with a lipophilic molecule such as fatty acid or by designing the drug to closely resemble a triglyceride, phospholipid or glyceride to increase likelihood of drug incorporation into the lipoprotein synthetic pathway.

For a drug to undergo lymphatic transport to an extent where bioavailability is improved a log P_{oct} value of >5 and triglyceride solubility of $\geq$ 50 mg / ml has been

found to be beneficial[97]. These parameters are only a guideline and other factors are also relevant such as the metabolic pathway of the drug and chemical stability. Influence from the type of lipid formulation used to deliver the drug upon the extent of lymphatic drug transport has also been considered. LCT has already been mentioned as the lipid type most likely to promote lymphatic absorption however further advantage may be gained by using lipolytic products i.e. long chain fatty acid and monoglyceride[90]. This approach eliminates the lipolysis stage thus reducing time required to initiate chylomicron formation with a resultant decrease in time available for drug to be absorbed into the portal blood.

Three different lipid vehicles, formulated to represent the physical stages of lipid digestion, were investigated to assess the extent of lymphatic absorption of the highly lipophilic free base form of halofantrine[98]. Results showed absorption to be more efficient as formulation dispersion status increased (micellar > emulsion > solution). However the model used eliminated any effects from gastric emptying and emulsification and when these were reintroduced using a conscious rat model[99] the type of dispersion had no effect upon the extent of halofantrine lymphatic uptake. Other evidence also suggests a role for *in vivo* bile mediated solubilization of drug to enhance transport by the lymph[100]. It therefore seems likely that as long as normal lipid processing can occur to reduce the lipid formulation to a micellar solution the long chain lipid used or the initial state of dispersion of the formulation will not influence the extent of drug lymphatic uptake.

1.4 Lipid-based formulations.

1.4.1 Stimulation of the lipid digestion cascade.

There is insufficient biological data available at present to be clear about the requirements of lipid formulations, although the studies already discussed in this chapter suggest the process of lipid digestion to be relevant. To develop a formulation which could benefit drug absorption via the various stages of physiological lipid processing it is necessary to know how much lipid is required to stimulate the lipid digestion cascade. Some of the early studies reporting increased bioavailability from

lipid-containing oral formulations were noted by Yamahira et al[101] to use large lipid volumes in excess of that practical for clinical use. They suggested a lipid dose equivalent to a 0.5 ml capsule to enable reasonable comparison of experimental results with those possible clinically.

Investigation into the minimum amount of emulsified corn oil necessary to maximize the uniformity and extent of absorption of griseofulvin[102] revealed a requirement of 4 g of corn oil contained within 10 g of an oil in water emulsion. These workers suggested drug absorption to be enhanced via a reduction in the gastric emptying rate and stimulation of gallbladder evacuation. Bioavailability of the poorly water-soluble steroid, danazol, from a hard gelatin capsule was noted to be increased three fold when given after a meal as opposed to dosage in the fasted state[103]. The use of a monoglyceride emulsion to administer the same dose of danazol increased plasma levels to those achieved when the capsule was given in the fed state. As bioavailability from the emulsion was independent of the presence or absence of food, a possible explanation is that the monoglyceride-based emulsion may have triggered a postprandial environment in the GI tract. The quantity of emulsion used to administer the dose of danazol was 30 g which is too large for use on a regular basis.

The large volume of lipid used in the above studies thus appears impractical from a pharmaceutical viewpoint, introducing problems of emulsion formulation and patient compliance with chronic usage. Further research is therefore required to assess if lower lipid volumes, suitable for encapsulation, could stimulate the lipid digestion cascade. However even if a significant proportion of lipid is found to be required, lipid-based formulations remain an option if the drug is needed only on a short term basis.

1.4.2 Constituents of lipid-based formulations.

Various types of lipid-based formulations exist; from a simple solution or suspension of drug in lipid, through to emulsions and more complex self-emulsifying and microemulsion systems. Due to the limited solubility of some hydrophobic drugs in lipids and to increase formulation dispersibility, other components such as co-solvents and surfactants are frequently included in a lipid formulation. The influence of these

constituents upon drug bioavailability may vary depending on the mechanism responsible for enhancement of drug absorption.

Certain surfactants have been demonstrated to render an otherwise digestible lipid unavailable to pancreatic lipase activity. When triglyceride is freely digested by lipase, drug preferentially partitioned in the lipid phase is proposed to be slowly released together with lipolytic products. The drug molecules and lipolytic products form a solubilized micellar phase with bile salts and lecithin which aids transport of drug to the site of absorption. A surfactant which prevents digestion would thus result in retention of drug in the lipid phase with an obvious disadvantage to drug bioavailability. Lack of or a reduction in the concentration of lipolytic products released could also decrease the solubilization capacity of the aqueous phase increasing the likelihood of precipitation of drug. The use of co-solvents in a formulation may also increase the possibility of drug precipitation upon release of the formulation into the aqueous GI fluid.

Surfactants have also been noted to increase the viscosity of GI fluid, a property shown mainly by polyethoxylated surfactants[104]. If surfactant concentration in a formulation was sufficient to mediate an increase in viscosity, drug absorption may be decreased due to a reduction in the diffusion rate of free drug molecules and micelles to the BBM; on the other hand drug absorption may be enhanced as an increase in viscosity may reduce the rate of gastric emptying.

When designing a formulation safety issues regarding the use of surfactants is a major consideration. Surfactants may be acutely damaging to the intestinal mucosa causing loss of epithelial cells from the surface, which although reversible, could in turn have a permeability enhancing effect on the intestinal membrane[85]. This may promote absorption of the surfactant molecules leading to possible toxicity through systemic mechanisms, in addition to increasing absorption of other non-related noxious materials in the GI tract.

Non-ionic surfactants are considered the least likely of surfactant types to induce toxicity. Several studies of homologous series of non-ionic surfactants[105, 106] have revealed toxicity, (measured by lethality through systemic effects), to increase in line with the non-polar nature of a surfactant and, in general, ability to act as an intestinal permeability enhancer[85]. However damage to the intestinal mucosa induced by a non-

ionic surfactant, Triton X-100, has been demonstrated to undergo restitution after only one hour[107]. In addition the GI tract has several defense mechanisms to prevent local damage such as the intestinal mucous layer, increased intestinal motility and enzymes, gut flora and buffering systems[85]. These together with the restitution capability of the intestinal mucosa may well be sufficient to overcome any membrane erosion caused by inclusion of a surfactant in a lipid-based formulation, as long as intervals between dosage are sufficient to enable time for repair.

An understanding of the influence of surfactants on the process of lipolysis appears desirable when designing a lipid-based formulation. Knowledge of other possible *in vivo* effects from constituents used in the formulation may also provide a means to enhance drug bioavailability and avoid potential problems. The benefits and adverse effects of surfactants and co-solvents on drug bioavailability and other issues related to formulation of lipid-based systems are addressed further in Chapter 5.

1.5 Overview of the investigations included in this thesis.

Hydrophobic drugs when dosed in a conventional tablet can exhibit limited bioavailability. In general terms the work in this thesis was intended to strengthen the argument for presentation of drugs in a lipid-based formulation when the above situation is observed. The activity of lipase towards a triglyceride substrate is central to the main mechanism proposed to be responsible for the enhancement of drug bioavailability from lipid-based formulations. Interest was therefore focused on the behaviour of lipase towards triglyceride under various conditions and in the presence of substances likely to be included in a lipid-based formulation.

Chapter 2

An *in vitro* model taken to represent the digestion of triglyceride by lipase in the duodenum had previously been developed[108] and was based on a titrimetric assay utilizing a stationary pH device (pH-stat). Chapter 2 involves examination and validation of various experimental conditions selected for use in the model, which is referred to throughout this thesis as 'the standard pH-stat assay'.

Chapter 3

A decrease in the activity of lipase towards triglyceride had been observed[108] when certain non-ionic surfactants were included in the assay. As surfactants are frequently used components of lipid-based formulations further knowledge regarding this phenomenon was desirable. Enzyme kinetic parameters can provide information regarding the mechanism by which enzyme activity is reduced when an inhibitory substance is present[109]. By determination of these parameters in the presence and absence of an inhibitory surfactant the mechanism by which surfactants are able to inhibit lipase may be revealed.

With this aim in view the activity of lipase towards triglyceride is quantified in Chapter 3 in terms of kinetic parameters, under conditions of the standard pH-stat assay. An attempt is also made to obtain the same parameters when a surfactant known to inhibit lipase activity is included in the assay system.

Chapter 4

Ideally a surfactant used in a lipid-based delivery system should fulfill the requirements of the formulation whilst having no influence on lipase activity. To enable selection of a surfactant which satisfies this criteria further knowledge regarding the behaviour of different non-ionic surfactant types towards lipase activity was required.

A range of non-ionic surfactant groups are screened for inhibitory behaviour towards lipase in Chapter 4 under conditions of the standard pH-stat assay. The results are analysed in relation to properties of the surfactants, namely the chemical structure and the HLB value, which describes the balance between the hydrophilic and lipophilic portions of a surfactant.

Chapter 5

The majority of surfactants tested in Chapter 4 decreased lipase activity under conditions of the assay, suggesting that a lipid-based formulation would probably include surfactants with some inhibitory behaviour towards lipase. Previous studies[108] had revealed certain surfactants with dominant lipophilic character were able to reduce the inhibition of lipase activity mediated by hydrophilic surfactants. This suggests a means by which a surfactant with inhibitory properties could be included in a formulation to achieve the level of dispersion required, in conjunction with a

lipophilic surfactant to overcome any consequential effect upon lipase. A range of lipophilic surfactants are screened in Chapter 5, using the standard pH-stat assay, for ability to overcome inhibition mediated by a hydrophilic surfactant.

Of considerable importance however is the relevance of lipase inhibition observed *in vitro* to the *in vivo* situation. To examine this aspect two lipid-based formulations of the same drug were designed to render one system digestible, when evaluated *in vitro*, and the other not. The development of these formulations is described in Chapter 5 followed by a discussion of results from an *in vivo* study where the formulations were administered to dogs. The *in vivo* study was performed by a contractor[110, 111].

Chapter 6

The main mechanism by which lipid-based formulations improve the bioavailability of hydrophobic drugs has already been suggested to involve solubilization of drug in bile salt and lecithin micelles[1]. If these micelles are able to greatly increase the solubility of a poorly water-soluble drug, stimulation of the lipid digestion cascade, and hence release of bile at the time of dosing could led to improved bioavailability. In Chapter 6 the extent to which solubilization may increase the solubility of a range of steroids was assessed under various conditions. The degree of solubility enhancement is examined in relation to the hydrophobic nature of the steroid, expressed via an experimentally determined parameter, log P_{HPLC}.

Chapter 7

The overall aim to encourage the use of lipid-based delivery systems is somewhat inhibited by the need to develop these formulations on an individual basis for each drug. Inability to predict the level of bioavailability enhancement likely to be achieved is another negative factor. The work in Chapter 7 involves adaptation of an existing mathematical model[112] which predicts the fraction of dose absorbed from a suspension formulation of a poorly water-soluble drug. The equations of the model were adapted to an emulsion system where drug is presented dissolved within lipid droplets. Results from the model are discussed in relation to which drugs and under what conditions an emulsion formulation may enhance bioavailability.

Chapter 2 - An *in vitro* model of lipolysis (the standard pH-stat assay).

2.1 Introduction

The use of a pH-stat to monitor the progress of enzymatic hydrolytic reactions where protons are liberated is a well established technique[113]. In the specific case of the pancreatic enzyme lipase, a titrimetric assay utilizing a pH-stat has become a standard method to determine the rate of substrate utilization[22, 114, 115].

Challis[116] used a pH-stat to investigate the lipolysis of lipid-based self-emulsifying systems by lipase and the method was further developed and validated by other workers at R P Scherer[108]. Their method enabled the extent of digestion of a lipid-based formulation, in a physiologically representative reaction medium, to be followed with respect to time. Comparison between the rate of digestion of various formulations by lipase could thus be made. The conditions for the model in terms of temperature, ionic strength and pH were selected to represent the *in vivo* situation.

The primary objective of the work described in this chapter was to examine and validate the conditions selected for use in the R P Scherer *in vitro* model. The effect of enzyme concentration and the pH of the reaction mixture on lipolysis of MCT by lipase were examined. An investigation was also performed to enable selection of a model triglyceride suitable for use throughout the study of lipolysis inhibition.

The set up, calibration, operation and maintenance of the pH-stat are described below, followed by an account of the methodology chosen for the *in vitro* model of lipolysis, referred to subsequently as the standard pH-stat assay. This assay procedure was used for all lipolysis experiments described in Chapters 3, 4 and 5. To help ensure validity of results obtained using the standard pH-stat assay several control experiments were also performed. These covered the issues of enzyme potency, substrate and enzyme stability, extent of triglyceride digestion and blank rates.

2.2 The standard pH-stat assay of lipolysis.

General principles of the assay.

A pH-stat assembly enables the progress of a reaction to be followed at constant pH, hence the device is particularly suitable for quantitative determination of enzyme reaction rates. The pH-stat operates by monitoring the electrode potential (expressed as pH), during a chemical reaction. The device is preset to an end-point pH value which corresponds to the constant pH at which the reaction is required to take place. Initially the reaction mixture has the same potential as the end-point pH however when the chemical reaction is started protons are liberated or taken up, with a resultant change in the potential. The pH-stat restores the reaction mixture to the set end-point pH by automatic continuous titration with acid or alkali. During the course of a reaction the volume of titrant used can be recorded as a function of time to create a progress curve of the reaction for kinetic analysis.

Relation of the assay to lipase activity.

A pH-stat is ideally suited to follow the lipolysis of triglyceride as the multi-phasic turbid nature of the emulsion does not interfere with the nature of the assay. Triglyceride is digested by the enzyme, pancreatic lipase and if the lipolytic reaction proceeds to completion a triglyceride molecule is split by ester hydrolysis into one monoglyceride and two fatty acids. The fatty acids release protons with a resultant drop in the pH. The pH-stat then releases a known volume of alkali (sodium hydroxide) to maintain the end-point pH value. The moles of sodium hydroxide used can be equated with fatty acid liberated to determine the percentage of triglyceride digested throughout the course of the reaction.

Optimization of the assay.

To help ensure precision of results with this type of assay the stirring rate of the reaction mixture must be kept constant and the electrodes should be calibrated. Absorption of carbon dioxide needs to be kept to a minimum and leakage of titrant from the burette tip should not occur. Another possible complication with the method is the existence of an unknown liquid junction potential[113]. This is created by different

diffusion rates of potassium and chloride ions into the reaction mixture from saturated potassium chloride present in the calomel electrode. The effect of any resulting diffusion potential upon the potential measured by the pH-stat was assumed to be minimal and therefore disregarded. This assumption was justified by determination of the apparent reaction rate before enzyme addition, known as the blank rate.

It is important to correctly set up the titrator unit of the pH-stat to ensure that the rate of titrant addition is sufficient to maintain the end-point pH throughout the reaction. If settings are too slow delays may occur in titrant addition whereas rapid addition of titrant can result in overshooting the required end-point pH value. Factors which affect selection of an appropriate titration rate include concentration of titrant, buffer capacity and size of the sample. Titrant addition is controlled by the proportional band selector, which determines the volume of titrant added and the pause between additions. This selector is set to a pH span prior to the end-point pH value. When the reaction mixture shows a reading corresponding to the pH setting on the proportional band selector, titrant delivery is gradually reduced as the end-point pH is approached. The setting of the proportional band selector is normally chosen so a reduction in titrant delivery starts when approximately 90 % of titrant needed to reach the end-point has already been added.

Preliminary experiments were performed to enable selection of the proportional band and titration speed settings. A titrant concentration of 1 M sodium hydroxide was found sufficient to ensure addition of a low volume to the reaction mixture thus minimizing dilution effects and possible reduction in reaction temperature. The buffer selected for use in the reaction mixture was tris-maleate which has the advantages of avoiding problems caused by other buffers such as calcium salt precipitation and absorbance of carbon dioxide. Tris-maleate can also be used over a pH range of 5.5 to 8.6[117] which covers the physiological pH values (4.8 to 8.2) found in the duodenum[118].

2.2.1 Materials

Tris-maleate buffer at pH 6.5

Trizma®-maleate (tris-maleate)	$C_4H_{11}NO_3 \cdot C_4H_4O_4$	50 mM	Sigma T3128
Sodium chloride	NaCl	150 mM	Sigma S9625
Calcium chloride dihydrate	$CaCl_2 \cdot 2H_2O$	5 mM	Sigma C3881
Sodium hydroxide	NaOH	40 mM	Fisons S/4880/60

Buffer was prepared by dissolving appropriate quantities of tris-maleate, sodium chloride and calcium chloride dihydrate in distilled water followed by addition of sodium hydroxide pellets to adjust to the required pH. As the pH of a buffer solution can vary significantly with temperature the buffer was brought up to experimental temperature before any final adjustments were made for volume and pH (using 1M NaOH solution).

Simulated bile solution

Taurodeoxycholic acid sodium salt	8 mM	Sigma T0875
L-α-phosphatidylcholine	1.6 mM	Sigma P9671
(60 % pure from fresh frozen egg yolk)		
to volume using tris-maleate buffer above.		

The simulated bile solution was prepared daily to minimize degradation of components. Appropriate quantities of sodium taurodeoxycholate (bile salt) and phosphatidylcholine (lecithin) were dissolved in tris-maleate buffer using a heated magnetic stirrer. After preparation simulated bile solution was stored at 4°C until required.

Triglycerides		
Tributyrin	$C_{15}H_{26}O_6$	Sigma T8626
Triolein	$C_{57}H_{104}O_6$	Sigma T7752
LCT	(Soya bean oil)	R P Scherer.
MCT	(Fractionated coconut oil as Miglyol® 812)	Hüls (UK) Ltd.

Additional materials		Supplier / Code No.
Porcine pancreatin		Sigma P1500
Porcine pancreatin		BDH 39029
Anti-foam A (100 % active silicone polymer)		Sigma A5633
Normadose hydrochloric acid	0.1 M	Prolabo 32/051/605
Normadose sodium hydroxide solution	0.1 M	Prolabo 32/067/600
Normadose sodium hydroxide solution	1 M	Prolabo 32/066/606
Standard pH 7 phosphate buffer		Fisons J/2850/17
Standard pH 4 phthalate buffer		Fisons J/2820/17
Standard pH 9.2 borate buffer		Fisons J/2870/15

2.2.2 Equipment

The pH-stat assembly

Radiometer pH-stat system ETS822 comprising of PHM82 Standard pH Meter, TTT80 Titrator, ABU80 Autoburette, and TTA80 Titration Assembly with stirrer (No.847-714).

Glass indicating electrode (No.G2040C)

Calomel pH reference electrode (No.K4040C)

All from Radiometer Analytical A/S, Copenhagen, Denmark.

Gallencamp water bath

B&T Circon pump

Water jacketed titration vessel (250 ml capacity)

The pH-stat was assembled in a stack system (from the top) of pH meter, titration assembly and auto burette with the stirrer unit holding the reaction vessel to the right.

The jacketed reaction vessel was connected via tubing to a pump unit contained in the water-bath. The pump allowed heated water to be circulated between the reaction vessel and the water bath. An integral thermoregulator on the water-bath maintained temperature with a separate thermometer suspended in the water bath to act as an external check.

Settings used for pH-stat assembly.

PHM82

Sensitivity	left as set after calibration (± 1 % absolute)
Temperature	37°C
Accuracy	± 0.01 pH at 25°C

ABU80

Speed	1.25 ml/minute
Volume	1/1
Man/auto	auto
Accuracy	imprecision of display value 0.005 ml
Accuracy in range 0 to 25 ml	titrant volume ± 7 µl ± 0.15 % of titrant volume

TTT80

Proportional band	0.05 pH units (accuracy ± 5 %)
Delay sec	pH-STAT
UP button	depressed (when end-point pH > reaction medium pH)
End-point	set at pH 6.5 unless otherwise stated for experiment
End-point accuracy	± 0.02 pH

Calibration of PHM82 standard pH meter.

To ensure accurate results from the pH meter a calibration procedure was necessary to check electrode function and response of the pH meter to the individual characteristics of each electrode. Calibration was performed on a daily basis before use of the pH-stat assembly for experimental work. The standard buffer solutions used

to calibrate the pH meter were selected according to the end-point pH of the assay. For an end-point pH below 7 buffer solutions of pH 4 and 7 were used, the pH 4 buffer was replaced by a buffer of pH 9.2 when the end-point value was set at ≥ pH 7. All pH measurements were taken at the same temperature as the experiments were to be performed (37°C) with the stirrer unit operational.

Calibration Procedure.

Electrodes were removed from storage solutions, rinsed with distilled water and located in the electrode head. With the pH meter sensitivity dial set at 100 % and the temperature dial to 37°C, the standard pH 7 buffer was left in the reaction vessel until a stable pH reading (± 0.01 pH units) had been displayed for 30 minutes. Using the buffer dial which alters parallel displacement the pH reading was adjusted to the manufacturers specified pH value at 37°C unless the correct reading was already shown. The pH was monitored for a further 15 minutes to ensure the reading remained stable, followed by rinsing of the vessel and electrodes with distilled water. The second buffer solution (pH 4 or 9.2, as appropriate) was placed in the vessel and left until a stable pH reading had been displayed for 15 minutes. If adjustment to the manufacturers specified value was necessary this was performed using the sensitivity dial which alters slope. A check was made to ensure the reading remained stable for 15 minutes. If so the vessel and electrodes were rinsed with distilled water and the pH 7 buffer solution was returned to the vessel. If the pH reading was within ± 0.01 pH units of the manufacturers specified value then calibration was complete. If the pH reading fell outside of these limits the whole procedure was repeated until results were within the specification.

2.2.3 Method for standard pH-stat assay.

The standard pH-stat assay described here was used for all lipolysis experiments; any modifications made to the assay for a particular investigation are noted under the corresponding description of the method.

Daily preliminary procedures.

Prior to starting the assay the burette unit was primed. Distilled water left in the burette for overnight storage was removed followed by flushing and refilling using titrant. This approach ensured no dilution of titrant and eliminated air bubbles. The titrant volume display was reset to commence from zero.

Pancreatin was weighed into 4 ml amber glass vials (fitted with Teflon lined rubber sealed lids), in amounts of 500 mg (+ 0.3 mg) and stored dry at 4°C until use. The required quantity of triglyceride was weighed into a vial of the same type as that used for pancreatin. If surfactant was also to be included in the experiment the triglyceride and surfactant were weighed into the same vial and thoroughly mixed. This technique will be referred to in future text as the premixed method.

For surfactants in a solid state at room temperature liquefaction was necessary to improve ease of handling and to aid dispersion into the reaction mixture. This was achieved by gentle heating of the complete storage container in a hot air cupboard until the surfactant was molten followed by thorough mixing to ensure homogeneity. After melting most surfactants remained in a liquid state after addition to triglyceride and were stored at 30°C until use. The above method was utilized for all surfactants listed in Chapters 3, 4 and 5 if solid / semi-solid at room temperature.

Assay procedure.

To start each assay 100 ml of simulated bile solution was required, the majority of which was placed in the reaction vessel. This was followed by the previously weighed sample of triglyceride or triglyceride with surfactant. The vial used to weigh the triglyceride was rinsed three times using the remaining simulated bile solution, with the rinsings added to the vessel followed by 10µl of anti-foam A to prevent foaming. Upon activation of the stirrer unit the reaction medium was left to equilibrate to 37°C (+ 0.5°C). When a stable pH reading had been shown for 10 minutes the mixture was titrated up to the end-point pH of 6.5, with a record made of the initial pH and titrant volume used. This step allowed adjustment for slight variations in starting pH values of the reaction mixture caused by different batches of simulated bile solution and properties of the triglyceride and surfactants.

Whilst the reaction mixture was reaching equilibrium temperature a vial of pancreatin was reconstituted by addition of 2 ml of distilled water. The resulting enzyme suspension was mixed by vortexing and left at 38°C for a pre-incubation period of 20 minutes. Following this period the pancreatin was re-suspended and 1 ml of the resulting enzyme suspension was added to the reaction medium which initiated the assay. The titrator and electronic timer were started simultaneously at this stage. The volume of titrant used was recorded manually using the timer at intervals of 1 minute for the first twenty minutes followed by readings every 5 minutes for a further 40 minutes.

Clean up and shut-down procedure.

To ensure no contamination occurred between assays the vessel underwent thorough washing to remove any remaining traces of enzyme and lipid using hot water and liquid detergent. The vessel was then rinsed with tap water four times followed by a final rinse using distilled water. Electrodes were cleaned by wiping with clinical tissue, followed by a distilled water rinse, and a final wipe with absolute alcohol 95 %. The reservoir was refilled with titrant.

If no further assays were to be performed during the same day the electrode head and stirrer were detached and scrubbed thoroughly using hot water and liquid detergent, followed by a distilled water rinse. The glass indicating electrode was placed in standard pH 4 buffer solution and the calomel pH reference electrode in a solution of saturated potassium chloride for overnight storage. The titrator reservoir was flushed twice using distilled water and then refilled with the same.

Processing of standard pH-stat assay results.

The assay gave readings of the volume of titrant used per unit time, which corresponded to protons released from fatty acid on a one to one molar basis. By assuming each triglyceride molecule underwent hydrolysis to form two fatty acids and a monoglyceride molecule, and that all free fatty acids were ionized, it was possible to determine the percentage of triglyceride digested out of the total initially present. For tributyrin the calculation was based on complete hydrolysis to three fatty acids and glycerol. Hydrolysis of tributyrin to completion was assumed as fatty acids with a

short chain length are known to be rapidly cleaved, especially *in vitro* in the presence of bile salts[119].

To perform the calculations the initial weight of triglyceride used in each experiment was adjusted for purity, if known, followed by determination of the total fatty acid available for possible hydrolysis by lipase. The volume of sodium hydroxide titrant recorded at each time point was converted to the corresponding concentration of neutralized free fatty acids. The percentage of triglyceride digested out of the total initially present was then determined from the concentration of free fatty acids. The percentage of triglyceride digested or amount of fatty acid neutralized was plotted against time to produce a progress curve of lipase activity referred to in future text as a digestion profile.

2.3 Assessment of lipase activity of pancreatin.

The source of lipase used in the standard pH-stat assay was pancreatin obtained from porcine pancreas. Pancreatin contains lipase, the enzyme responsible for hydrolysis of triglyceride in the duodenum together with the required cofactor, colipase. Porcine pancreatin was selected as a substitute for human pancreatin as both porcine pancreatic lipase and colipase have been described as displaying a high degree of sequence homology to the human derived source[30, 120] with a similar pH-activity profile[121].

Due to the crude, variable nature of pancreatin certain minimum standards of activity are set. For batch I to III of pancreatin used in this study the supplier specified a minimum lipase activity of at least to the United States Pharmacopoeia (USP) specification[122]. The supplier of batch IV pancreatin stated compliance with the British Pharmacopoeia (BP) 1968 standard for pancreatin[123] which was based on assessment of pancreatin activity towards triacetin. Martindale[124] provides a conversion factor for the BP 1968 standard to the BP 1980 standard. The USP and BP 1980 standards are both based on the rate of hydrolysis of olive oil and the potency of one USP unit is stated as approximately equivalent to the BP unit of lipase activity[124]. Based on these figures the minimum activity of batch IV pancreatin per mg

is 6.15 units compared to batch I to III pancreatin with a minimum activity of 2 units per mg.

As no information was available regarding maximum activity a more specific measure of lipase activity was required to ensure equivalence between batches of pancreatin used throughout this thesis. Pancreatin activity was thus assessed using a tributyrin substrate. Tributyrin is an ideal substrate to measure lipase activity[41, 125] as it yields completely ionized water-soluble products under alkaline conditions (pH 8 to 9). Although tributyrin is hydrolyzed between two to ten times faster than long chain triglyceride a linear reaction rate is normally maintained for a sufficient time period to assess maximum activity. Tributyrin has additional advantages over long chain triglycerides in that it emulsifies spontaneously in dilute sodium chloride solution removing the need for emulsifier. Inhibition of lipase activity due to emulsifier at the substrate / water interface is thus avoided as is any interference from bile salts which can modify the pK values of essential ionizable groups on the enzyme and shift the optimum pH of lipase.
For each batch of pancreatin (batch I-IV), maximum lipase activity was assessed according to the procedure described below.

2.3.1 Method

Lipase activity of pancreatin was standardized titrimetrically at pH 8.5 and 25°C using a continuous pH-stat technique and a tributyrin emulsion (3.6 g tributyrin in 100 ml buffer containing 50 mM tris-maleate pH 8.5, 5 mM calcium chloride dihydrate and 150 mM sodium chloride). The pancreatin was prepared as a suspension in distilled water (150 mg / 3 ml), mixed by vortexing and incubated for 20 minutes at 25°C followed by a second thorough mixing before use.
100 ml of tributyrin emulsion and 25 mg of pancreatin (0.5 ml of suspension) were used for each assay. These reagents were stirred continuously whilst the pH was maintained constant with 1 M sodium hydroxide. The volume of titrant used was recorded every 15 seconds for 12 minutes. The highest volume of titrant used over

four consecutive readings corresponded to the maximum catalytic potential of the enzyme per minute under conditions of the assay.

2.3.2 Results

The enzyme activity determined for each batch of pancreatin is shown in Table 2.1. One tributyrin unit (TBU) of lipase activity is defined as the liberation of one micromole of butyric acid from a substrate of tributyrin per minute under conditions of the assay. For the purpose of this work the specific activity of pancreatin (number of TBU per mg of pancreatin) was used to express the potency of the enzyme extracts.

Pancreatin			Specific activity of pancreatin (Maximum No. of TBU per mg per minute).
Batch	Supplier	Batch No.	
I	Sigma	13H0347	9.6
II	Sigma	14H0877 obtained 10/95	8.0
IIa	Sigma	14H0877 obtained 6/96	8.8
III	Sigma	53H0442	8.0
IV	BDH	Not stated	9.2

Table 2.1 Specific activity determined for each batch of pancreatin used in the standard pH-stat assay.

A minimal difference in lipase activity was noted between pancreatin batches in terms of the number of TBU per mg of pancreatin however the significance of the parameter of TBU was unknown in relation to the rate of digestion of medium and long chain triglycerides intended for use in the standard pH-stat assay. To assess the difference between pancreatin batches when acting upon MCT (1 g) the standard pH-stat assay was performed using each batch of pancreatin. Results from this investigation are shown in Figure 4.8 (section 4.4.3) where the difference in activity between the various batches of pancreatin was concluded to be minimal.

2.4 Selection of a triglyceride substrate.

The main criteria for selection of a triglyceride suitable for use in the standard pH-stat assay was availability to the hydrolytic activity of pancreatin. This was to ensure that any inhibition of enzyme activity due to the presence of surfactant could easily be detected. A preliminary investigation was performed to assess the availability of a range of triglycerides to hydrolysis by pancreatin. A known weight of triglyceride (approximately 0.45 g) was digested under conditions of the standard pH-stat assay for a period of 60 minutes. For certain triglycerides the digestions were replicated.

2.4.1 Results and discussion.

The quantity of triglyceride digested after 60 minutes is shown in Table 2.2 for each triglyceride, as a percentage of the total triglyceride present.

Triglyceride	Dominant fatty acid/s	Purity %	Molecular weight	% total triglyceride digested
Tributyrin	$C_{4:0}$	99	302.4	98 (at 66 minutes)
Triolein	$C_{18:1}$	65	885.4	56, 54, 53, 61, 55.
LCT	$C_{18:1}$ 25 %	-	870.86*	40, 38.
	$C_{18:2}$ 51 %			
MCT	C_8 60 %	99.7	503.6#	92, 90.
	C_{10} 40 %			

Table 2.2 Percentage of the total triglyceride digested within a 60 minute period, for a range of triglycerides.
* Calculated using average fatty acid composition for Soya bean oil[126].
# Calculated using average fatty acid composition for Miglyol® 812[127].

To enable comparison between experiments the results were expressed as a percentage of the total triglyceride present. The various triglycerides were assayed at equal weight therefore use of a percentage scale eliminated complications introduced by their different molecular weight values and hence molar concentrations.
From examination of Table 2.2 the MCT, fractionated coconut oil was chosen for further investigations due to adequate hydrolysis in the assay system, pharmaceutical acceptability, high standard of purity, low cost and availability with specified proportions of the component fatty acids.

2.5 Effect of pH on enzyme activity.

In common with other enzymes lipase activity has been demonstrated to vary with pH[121]. The optimum pH for lipase activity will depend upon conditions of the reaction medium such as the concentration of bile salts[128] and ionic strength[129]. A pH of 6.5 was proposed for use with the standard pH-stat assay on the basis that it was typical of the fasting duodenal pH in healthy subjects[130].
Determination of the optimum pH for pancreatin activity under conditions of the standard pH-stat assay would enable assessment of the degree of enzyme activity remaining at the chosen pH of 6.5. The maximum velocity of pancreatin activity towards a substrate of MCT was therefore assessed over a pH range of 5 to 9.

2.5.1 Method

A series of digestion profiles were performed under conditions of the standard pH-stat assay, using a substrate of 1 g of MCT. The end-point pH values used ranged from 5 to 9 at 0.5 increments. Simulated bile solution for each experiment was prepared using tris-maleate buffer of a pH value corresponding to the particular reaction end-point pH. This was achieved by preparing a concentrated solution of tris-maleate buffer with sodium hydroxide omitted. The buffer solution was then adjusted to the required pH by adding a sufficient quantity of dry sodium hydroxide and made up to volume. Before use simulated bile solution was equilibrated to 37°C and the pH rechecked to allow any final adjustments with 1 M sodium hydroxide solution if required.

2.5.2 Results and discussion.

A digestion profile of product formed, (expressed as millimoles of fatty acid released), with time was produced in duplicate for each end-point pH. The gradient of the initial linear portion of each digestion profile was used to determine the initial velocity of the reaction via linear regression, after ensuring no lag phase (described in section 3.3.3) was present at the start of the reaction.

Figure 2.1 demonstrates the dependence of pancreatin activity on pH of the reaction mixture when hydrolyzing a substrate of MCT. The optimum pH for pancreatin activity under conditions of the standard pH-stat assay was approximately 7.5, indicated by the apparent maximum initial velocity observed of 0.345 mmol min^{-1} (Figure 2.1). For a pH end-point of 6.5 the initial velocity fell to 0.19 mmol min^{-1} which represents a 45 % reduction in enzyme activity compared to the apparent maximum possible.

When the digestion profiles were examined in terms of the percentage of MCT digested after 60 minutes (results not shown) this was also noted to vary with pH. For a pH of 6.5, 83 % of the total triglyceride present had been digested whereas at pH 7.5 this figure increased to 103 %. When using the optimum pH for pancreatin activity (pH 7.5) some triglyceride appears to have undergone complete hydrolysis to fatty acids and glycerol. This may be explained by the high rate of enzyme activity coupled with a high degree of emulsification conferred by the presence of bile salt. Although triglyceride hydrolysis has been stated as likely to proceed to completion *in vitro*, this situation would be unlikely *in vivo* due to mechanisms available for absorption of monoglycerides[119].

Although the reaction kinetics of pancreatin are dependent upon pH, under conditions of the standard pH-stat assay (a pH of 6.5) pancreatin had sufficient activity to enable assessment of hydrolytic behaviour towards various substrates.

An explanation for the observed decrease in enzyme activity away from the optimum pH of 7.5 may involve change in the ionization status of ionic groups present in the enzyme or substrate[117]. If these ionic groups are not in the required ionic form, alteration in conformation of the active site, enzyme / substrate binding or catalytic ability may occur with a resultant decrease in enzyme activity or enzyme inactivation. In the case of porcine pancreatic lipase the catalytic triad of the active site of the enzyme is known to be composed of ionizable amino acid residues, serine, aspartic acid and histidine[26]. Early workers[41] with porcine pancreatic lipase suggested a requirement for the histidine residue to be ionized in order to achieve the maximal rate of hydrolysis of tributyrin. They quoted a pKa value for histidine in their system of 5.8 although the exact pKa values can be influenced by the environment of the enzyme.

The pH of the environment in which an enzyme is present can cause irreversible enzyme inactivation and this is known to occur with porcine pancreatic lipase at pH values lower than 5[131]. As lipase activity in Figure 2.1 varies when pH is raised above 5, the ionic state of residues in the active site is suggested as more likely to be responsible. This could be confirmed by further experimentation involving pre-incubation of the enzyme for a period equal to the length of the assay at various pH values before commencing digestion at pH 7.5. If the reaction rate was reduced this would indicate pH related enzyme inactivation. However as the work described here was carried out to investigate the appropriate nature of the assay conditions rather than to characterize the enzyme this investigation was not performed.
A further consideration is the possible influence of the ionic strength of the buffer, (used to prepare the simulated bile solution), upon the pH activity profile (Figure 2.1). The tris-maleate buffer contained varying concentrations of sodium hydroxide to adjust the pH resulting in higher ionic strength for the more alkaline solutions. To correct for this each solution could have been brought to a constant ionic strength by adding the appropriate quantity of a neutral non-inhibitory salt, however this would have further altered reaction conditions from those of the standard pH-stat assay.

2.6 Selection of enzyme concentration.

For an enzyme reaction taking place in solution, the initial velocity of the reaction should be proportional to the enzyme concentration (Equation 2.1)[109].

$$v = k[E] \qquad \text{Equation 2.1}$$

If this is the case a linear relationship should be obtained which passes through the origin indicating zero activity at zero enzyme concentration. If the relationship deviates from linearity this is usually a result of the assay system although it can sometimes be due to a property of the enzyme[132].
The relationship between pancreatin concentration and initial velocity was examined to ensure no interference was present from the standard pH-stat assay system. This approach also enabled the enzyme concentration selected for use in the standard pH-

stat assay (250 mg of pancreatin) to be examined in terms of initial velocity compared to that shown by higher quantities of pancreatin.

2.6.1 Method

A series of digestion profiles were produced under conditions of the standard pH-stat assay using a fixed substrate concentration of 1 g of MCT and varying concentrations of pancreatin. As a quantity of 250 mg of pancreatin had been proposed for use in the standard pH-stat assay, the enzyme concentrations chosen for use in this investigation spanned a range from 62.5 mg up to 500 mg. The enzyme suspension was prepared in the normal manner with adjustments for strength made by varying the volume of distilled water. This ensured minimal variation in the volume of enzyme suspension added to each assay (between 0.5 to 1.25 ml). The series of digestion profiles were performed for both batch I and II of pancreatin.

2.6.2 Results and discussion.

The initial velocity of each digestion profile was determined as described in section 2.5.2 and is shown as a function of enzyme concentration in Figure 2.2 for batch I and II of pancreatin. A linear relationship between initial velocity and pancreatin concentration was shown by batch II of pancreatin ($r^2 = 0.9985$) if the result obtained using 500 mg of pancreatin was disregarded.

As the velocity of a reaction is known to vary with substrate concentration, the initial velocity should be determined from the gradient of the digestion profile when only 5 % or less of the substrate has been used[133]. This approach ensures that initial velocity is a true value and not limited by substrate availability. For the high pancreatin concentration of 500 mg (batch II) 13.35 % of MCT had been utilized 2 minutes after commencing the assay. This reaction was too fast to enable accurate measurement of the true initial rate which probably accounts for deviation of this result from the linear relationship displayed by the lower enzyme concentrations.

The data obtained using batch I pancreatin indicated an apparent maximum initial velocity, first evident at an enzyme concentration of 250 mg (Figure 2.2). Non-

linearity between initial velocity and concentration of batch I pancreatin may be due to error in determination of initial velocity. For profiles produced using batch I pancreatin intervals of 5 minutes were used to record titrant volumes which resulted in high substrate utilization (11 to 37 %) before the first volume was recorded. These readings were too widely spaced to give accurate results with substrate concentration probably limiting initial velocity. For profiles produced using batch II pancreatin titrant recordings were taken every minute for the first 20 minutes and thereafter every 5 minutes which appeared to reduce this source of experimental error.
When considering the suitability of 250 mg of pancreatin for use in the standard pH-stat assay, concentrations above 250 mg gave a faster initial rate of digestion. However the total percentage of triglyceride digested after 60 minutes remained the same with approximately 80 % digestion shown by both batches at concentrations of 250 mg and above (results not shown). Therefore 250 mg of pancreatin was concluded to be appropriate for use in the standard pH-stat assay due to the smooth digestion profile produced which displayed the maximum extent of digestion possible at pH 6.5 within the 60 minute assay.

2.7 Stability of pancreatin and other assay components.

To ensure validity of results from the standard pH-stat assay, control experiments were performed to assess stability of all assay components throughout the duration of the assay.

2.7.1 Pancreatin

In the case of pancreatin, enzyme activity and stability was assessed with respect to time and in the presence of other assay components. To examine the intrinsic activity of pancreatin over the total assay period of 80 minutes pancreatin was reconstituted in the normal manner and then left for pre-incubation periods of 0, 40 and 60 minutes instead of the usual 20 minute period. Immediately following the period of pre-incubation the pancreatin solution was used to digest 1 g of MCT under conditions of the standard pH-stat assay.

To determine if any of the assay components affected enzyme stability, experiments were performed whereby instead of reconstituting the enzyme with distilled water as in the standard pH-stat assay, the enzyme was reconstituted using buffer, simulated bile solution and simulated bile solution mixed with a non-ionic surfactant (Cremophor 0.5 % $^{w}/_{v}$). The pancreatin solution was then pre-incubated for 20 minutes and used to digest 1 g of MCT under conditions of the standard pH-stat assay.

Results and discussion.

The dependence of pancreatin activity on the duration of the pre-incubation period used for the pancreatin suspension is illustrated in Figure 2.3. Although the rate of digestion was slightly reduced as the pre-incubation period was increased, after 40 minutes all profiles displayed a similar percentage of triglyceride digestion (Figure 2.3).

When the time taken to digest a specified percentage of MCT was determined from the digestion profiles in Figure 2.3 and displayed as a function of pre-incubation time (Figure 2.4), the difference in time taken for digestion can be seen to be minimal. The results showed enzyme activity to be virtually unchanged when using a pre-incubation period of 20 minutes compared to the digestion rate when the pre-incubation stage was omitted (Figure 2.4).

The effect of reconstitution of pancreatin with solutions containing various assay components as opposed to the normal medium of distilled water is illustrated in Figure 2.5. The resulting digestion profiles (Figure 2.5) suggest close contact of enzyme solution during pre-incubation with buffer components, bile salts, lecithin and non-ionic surfactant did not inhibit subsequent pancreatin activity towards a substrate of MCT. This result could be used to support the assumption that pancreatin was also stable when in the presence of the same components in the bulk reaction vessel.

2.7.2 Substrate of MCT.

An investigation was designed to examine the stability of the substrate (MCT) and simulated bile solution when combined together over the total assay period. MCT (1

g) was mixed with simulated bile solution and left in the reaction vessel with the stirrer unit operational for 80 minutes to represent the pre-incubation period and duration of the assay. The reaction mixture was then digested under conditions of the standard pH-stat assay.

Results and discussion.

Figure 2.6 demonstrates the stability of MCT and simulated bile solution when combined and left for a period equivalent to the total duration of the assay before commencement of digestion. Pancreatin activity towards MCT which had undergone a prolonged stirring period of 80 minutes and the same substrate under conditions of the standard pH-stat assay was identical. The conclusion can therefore be drawn that MCT remains stable in the presence of simulated bile solution throughout the total assay period.

2.7.3 Extent of triglyceride digestion.

When digesting MCT (1 g) under conditions of the standard pH-stat assay the total percentage of triglyceride converted into monoglyceride and two fatty acids did not exceed 80 % (Figures 2.3, 2.5-6). The reason for this was unknown although enzyme activity or stability may decrease as the concentration of lipolytic products in the vessel is increased.

Bile salts and lecithin have been suggested to decrease fatty acid concentration within an *in vitro* system by solubilization of lipolytic products into mixed bile salt and lecithin micelles[134]. Failure to achieve total triglyceride digestion in the *in vitro* standard pH-stat assay may thus relate to insufficient concentrations of bile salt and lecithin in the simulated bile solution to solubilize all fatty acids released.

An investigation to determine if the concentration of bile salt and lecithin used in the standard pH-stat assay was limiting the extent of triglyceride digestion was therefore performed. Simulated bile solution was prepared in the normal manner to contain double (bile salt 16 mM, lecithin 3.2 mM) and half (bile salt 4 mM, lecithin 0.8 mM) the concentrations of bile salt and lecithin present in the standard simulated bile

solution. The resulting simulated bile solutions were then used to perform a digestion of 1 g of MCT under conditions of the standard pH-stat assay.

To determine if enzyme activity was reduced with time due to presence of lipolytic products in the reaction mixture 3g of MCT was digested under conditions of the standard pH-stat assay for 120 minutes. Fresh enzyme solution containing 250 mg of pancreatin (prepared in the normal manner) was added to the reaction mixture 120 minutes after the start of the assay and then left to digest for a further 20 minutes.

Results and discussion.

From Figure 2.7 it can be seen that altering the concentrations of bile salt and lecithin present in simulated bile solution does not alter the extent of triglyceride digestion or the rate of pancreatin activity towards 1 g of MCT. This result suggests that bile salt concentration is not a limiting factor in the standard pH-stat assay in terms of desorption of lipolytic products from the interface and subsequent solubilization into micelles.

When using an increased quantity of MCT (3 g) only 52 % of the total triglyceride present was digested after 120 minutes (Figure 2.8). When fresh pancreatin (250 mg) was added to the reaction vessel 120 minutes after assay commencement a further 4 % of triglyceride was digested over a 20 minute period.

From comparison with the usual total percentage of triglyceride digestion (80 %) achieved with 1 g of MCT, Figure 2.8 suggests that as substrate concentration is increased the total percentage of triglyceride digested is reduced. Lack of substrate is therefore not responsible for the decline in enzyme activity which also seems unlikely to be a result of enzyme degradation with time as fresh enzyme also showed reduced activity. The inhibition of further pancreatin activity towards the remaining triglyceride is probably a form of product inhibition caused by a higher fatty acid concentration in the vessel than is reached with 1 g of MCT.

A final point for consideration is the percentage of the liberated fatty acids in an ionized state at the pH of the reaction mixture. Only fatty acids in an ionized state will be available for detection by a titrimetric method, with the percentage of ionized fatty acid dependent upon fatty acid pK value. Long chain fatty acids have been noted to be incompletely ionized at slightly acidic pH values[125] such as those found in the

duodenum and this is also likely to be the case for the medium chain fatty acids present in this system.

The pK value of long chain fatty acids has been demonstrated to decrease when calcium ion and ionic strength of the system was raised[134]. To increase the percentage of fatty acids ionized when titrated under conditions of the standard pH-stat assay the calcium ion concentration and / or ionic strength of the simulated bile solution could have been increased to depress pK values. However this approach was inappropriate due to the need to represent physiological conditions.

A correction factor could be used to determine the actual reaction rate from the pH-stat responses by calculation of the percentage of fatty acids ionized using the medium chain fatty acid pK value[134]. Determination of pK values under conditions of the standard pH-stat assay was not performed as results from digestion profiles were required for comparison of the influence of surfactants on digestion rather than absolute values of digestion. The adequate percentage of MCT digestion recorded (80 % at pH 6.5) without the application of a correction factor was considered sufficient to make this comparison.

2.8 Investigation of blank rate.

A blank rate is the existence of an apparent rate of reaction within an assay system which is not due to the monitored reaction[132]. If the blank rate is significant its source needs to be understood and the extent quantified so that appropriate corrections can be made. In the case of the standard pH-stat assay, volume of titrant recorded should be directly related to release of fatty acid from triglyceride with no interference from a blank rate.

To examine the system for the existence of a blank rate the standard pH-stat assay was performed with various components eliminated in turn. These control experiments revealed a blank rate to occur only when enzyme was present in the system. Upon addition of pancreatin to tris-maleate buffer an immediate drop in pH was seen followed by no further release of titrant over a 60 minute period. The initial drop in pH was corrected within a couple of seconds by titration of 0.02 ml sodium hydroxide (1 M) and this volume remained constant for all experiments performed. A

possible reason for the reaction rate detected could be acidity of the enzyme suspension or endogenous substrate material contained within it.

Upon addition of pancreatin to simulated bile solution a blank rate totalling 0.05 ml of sodium hydroxide 1 M occurred during the 60 minute assay which was slightly higher than the blank rate observed for pancreatin with tris-maleate buffer. This suggests a component of the simulated bile solution, most probably lecithin, to be responsible for the increase in blank rate. The knowledge that pure porcine pancreatic lipase, in the presence of bile salts, is able to hydrolyze the *sn*-1 ester bond of egg lecithin[131] gives further weight to this theory.

Further experimentation revealed the blank rate to be constant therefore correction could be made by subtraction from the assay results. In the case of the kinetic studies performed in this work subtraction would be inappropriate as the blank rate obtained is part of the integral enzyme activity, thus to discount it would result in underestimation of the enzyme activity. For the non-kinetic work subtraction was also considered unnecessary due to the minimal and constant nature of the blank rate compared to the overall titrant volume recorded.

2.9 Conclusions

The standard pH-stat assay of lipolysis described in this chapter was considered suitable to determine the availability of triglyceride to lipase, present in the assay in the form of porcine pancreatin. The optimum pH for pancreatin activity under conditions of the assay was pH 7.5, although a value of 6.5 was used for the standard pH-stat assay as this was more representative of conditions in the duodenum. The percentage of MCT hydrolyzed by pancreatin under conditions of the standard pH-stat assay was sufficient to allow observation of possible inhibition of enzyme due to the presence of surfactants in future work.

Components of the assay system remained stable for an assay duration of 60 minutes. The concentration of bile salt and lecithin used in the simulated bile solution had little influence on the rate and extent of triglyceride digestion within the range tested. Distortion of results from the standard pH-stat assay due to blank rates were minimal

and constant between assays thus alterations to results to account for these were not required.

Figure 2.1 Effect of pH on the initial velocity of pancreatin-catalysed digestion of MCT.

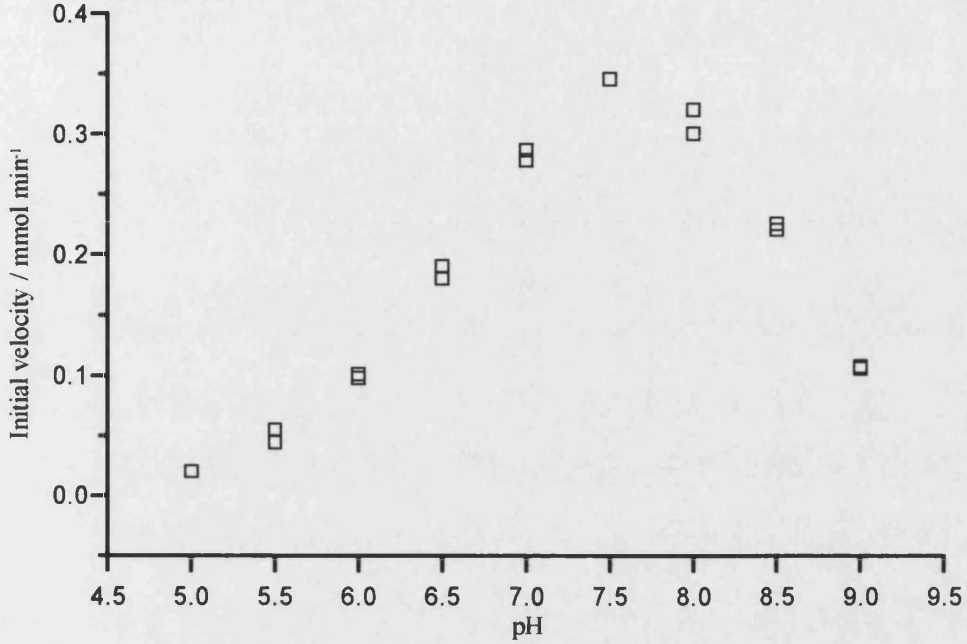


Figure 2.2 Effect of pancreatin concentration on the initial velocity of pancreatin-catalysed digestion of MCT.

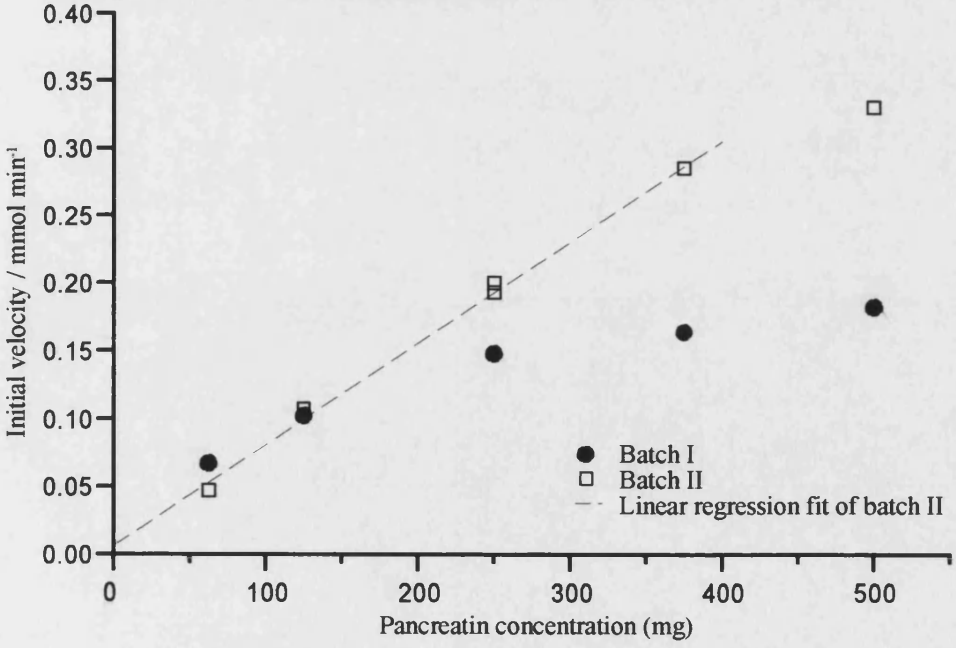


Figure 2.3 Effect of pancreatin pre-incubation time on the rate and extent of pancreatin-catalysed digestion of MCT.

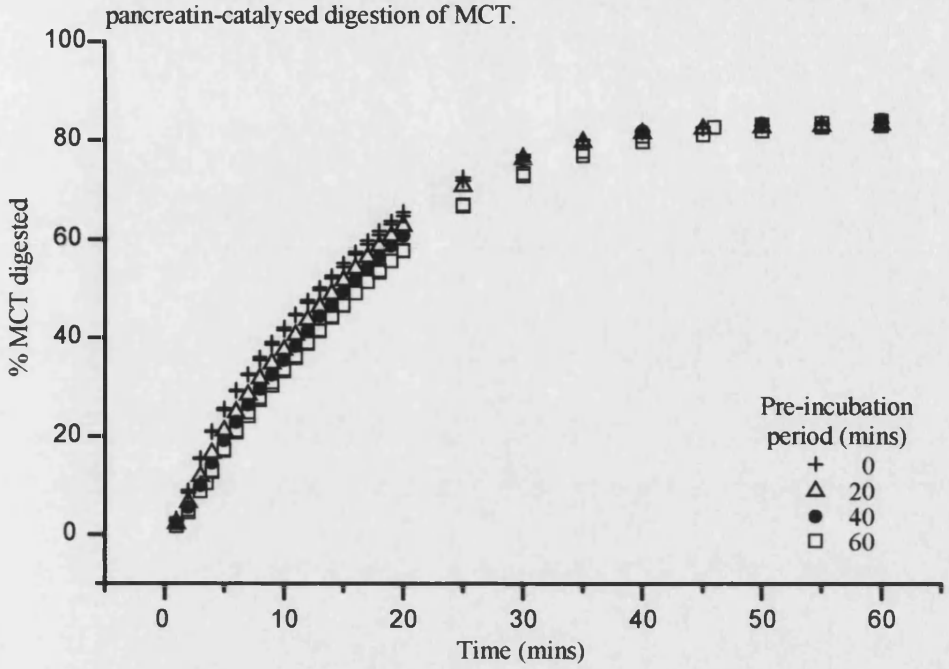


Figure 2.4 Effect of pancreatin pre-incubation on time taken to digest a specified percentage of MCT.

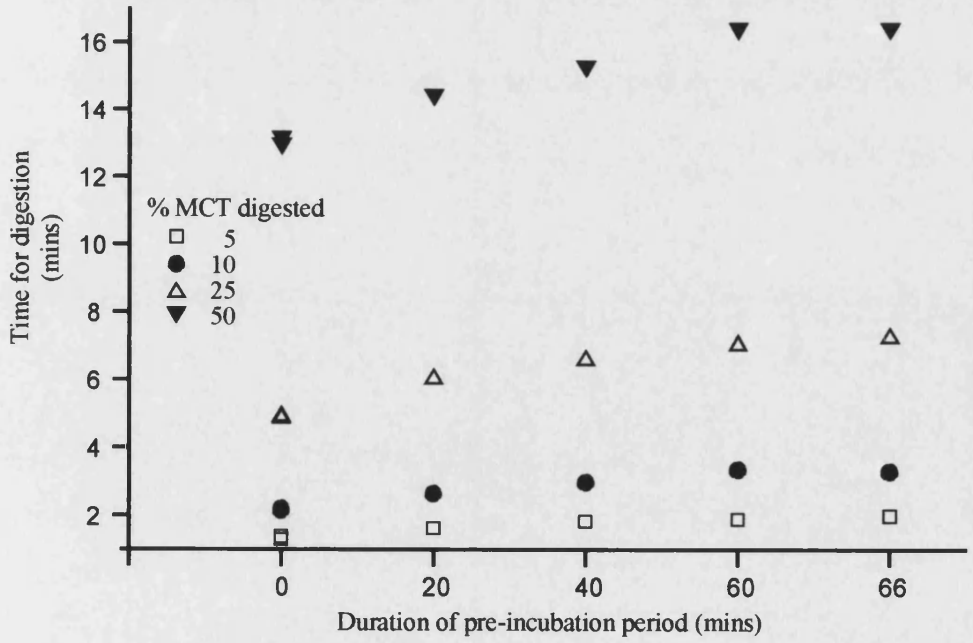


Figure 2.5 The effect of method used to reconstitute pancreatin on rate and extent of digestion of MCT.

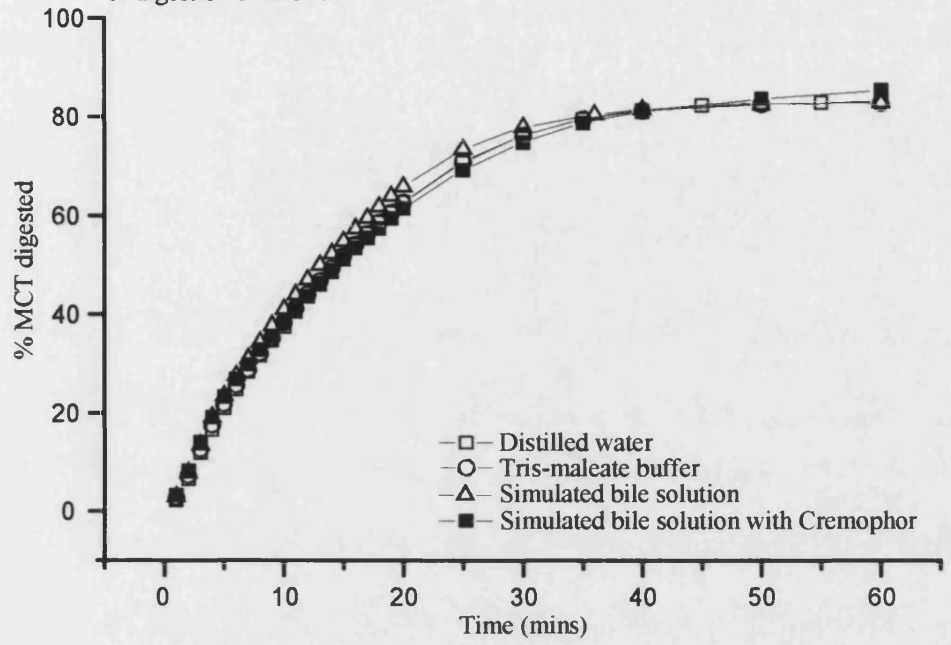


Figure 2.6 Stability of substrate and simulated bile solution throughout the duration of the standard pH-stat assay.

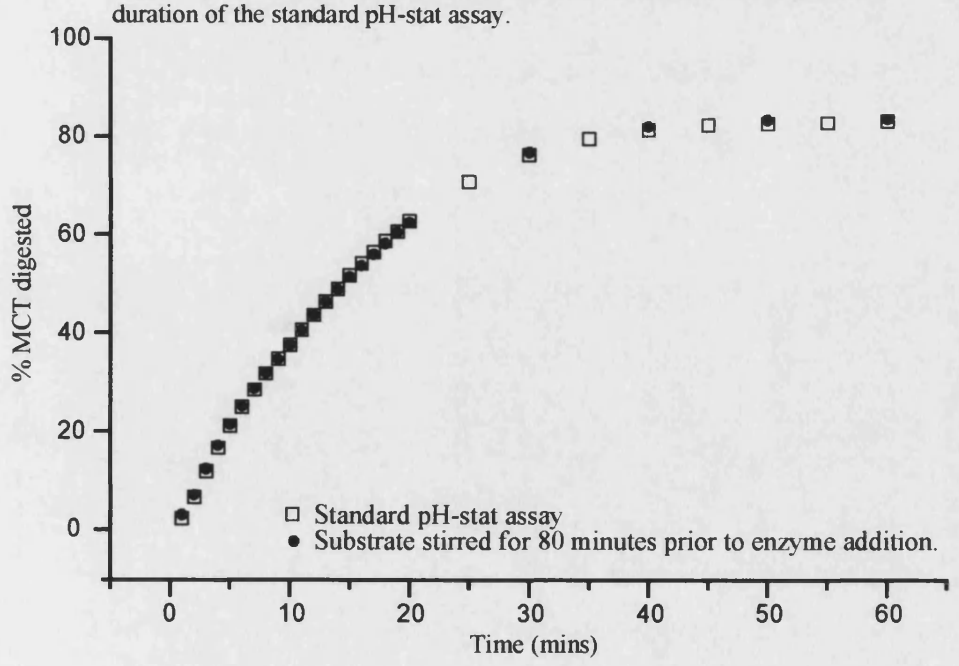


Figure 2.7 Influence of bile salt and lecithin concentration on the rate and extent of pancreatin-catalysed digestion of MCT.

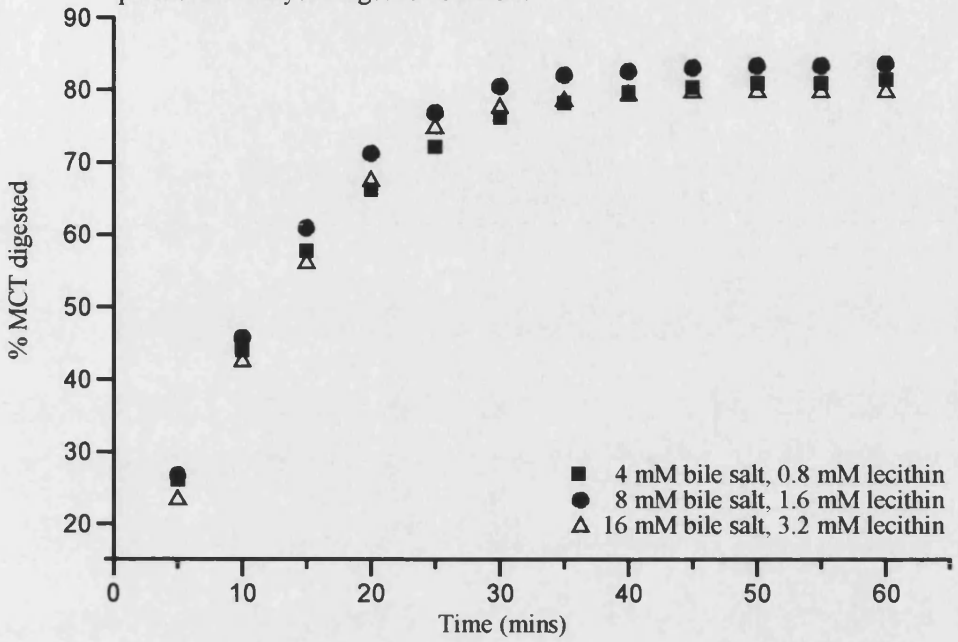
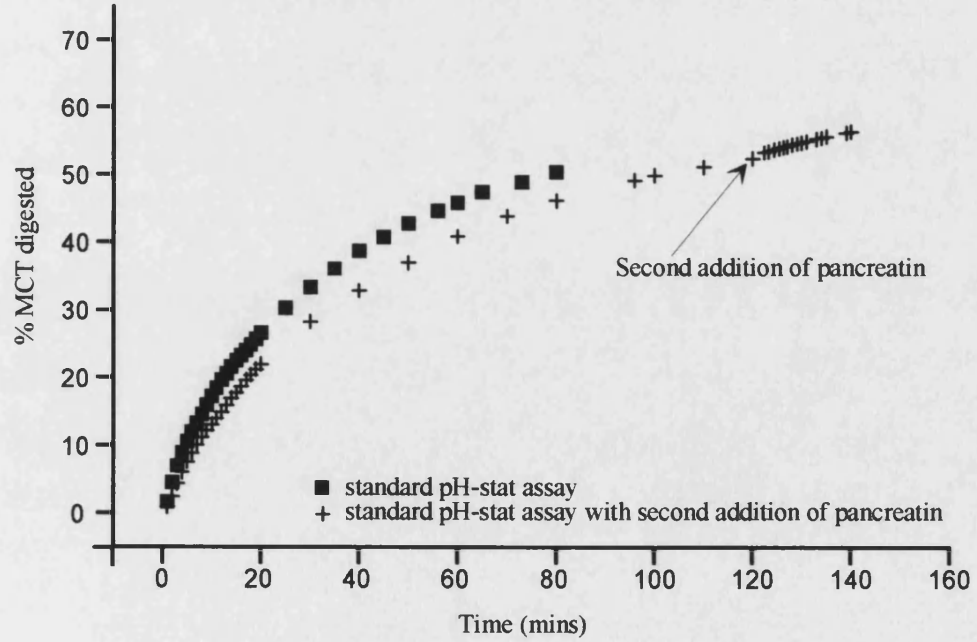


Figure 2.8 Rate and extent of pancreatin-catalysed digestion of 3 g of MCT.



Chapter 3 - Determination of the kinetic parameters K_m and V_{max} for pancreatin acting upon MCT alone and in the presence of the surfactant, Cremophor RH40.

3.1 Introduction

Lipid-based formulations commonly include one or more surfactants to enhance dispersion of the formulation in the GI fluid. Certain surfactants have been found to be inhibitory towards pancreatin activity[108]; a property which could result in prevention of *in vivo* digestion of lipid in the formulation. As lipid digestion is proposed to be an important mechanism of enhanced hydrophobic drug bioavailability from lipid-based formulations[1] use of surfactants which do not inhibit pancreatin is to be preferred. To enable avoidance of surfactants with potent properties of enzyme inhibition a means of classifying surfactants according to inhibitory behaviour in this respect would be advantageous.

Pancreatin activity towards a substrate of MCT can be characterized in terms of the kinetic parameters K_m and V_{max}. These parameters, derived from the Michaelis-Menten relationship[135], describe the relationship between the initial velocity of pancreatin activity upon MCT and the concentration of MCT present. K_m and V_{max} are of a constant value for a particular enzyme acting under specified reaction conditions, therefore when a surfactant capable of interfering with pancreatin activity is included with MCT in the reaction system K_m and V_{max} values will change accordingly.

The change in parameter values reflects the altered relationship between initial velocity and MCT concentration due to the presence of an inhibitory surfactant. The effectiveness of the inhibitor can be defined by an inhibitor constant (k_i)[109]. Therefore determination of k_i values *in vitro* for each surfactant would provide guidance with regards to their likely inhibitory potency towards lipase *in vivo*. This information would be useful when selecting a surfactant for inclusion in a lipid-based formulation.

A discussion of special factors for consideration when performing kinetic studies with an enzyme such as lipase, which acts at the interface of an insoluble substrate is given in section 3.1. Section 3.2 provides a description of the theoretical basis of the Michaelis-Menten relationship. This is followed by determination of the kinetic parameters K_m and V_{max} for pancreatin acting upon MCT under conditions of the pH-stat assay. The second stage of the investigation (section 3.3-3.6) involves an attempt to determine K_m and V_{max} when a non-ionic surfactant, (Cremophor RH40, referred to hereafter as Cremophor) known to inhibit pancreatin was included in the assay.

3.1.1 Kinetic studies involving enzymes which act at an interface.

When investigating the kinetic behaviour of lipase it is necessary to bear in mind that lipolysis occurs within a heterogeneous system whereas the concepts of enzyme kinetics are based on an homogeneous system. An homogeneous system is one in which the enzyme and substrate are freely soluble in an aqueous environment with enzyme specificity solely related to the chemical nature of the substrate.
Within a system where lipolysis is taking place a soluble enzyme acts upon an insoluble substrate at a lipid-water interface. The nature of lipolysis means that the specificity of the enzyme is related to the state of the substrate at the interface as well as the affinity of the active site of the enzyme for a single substrate molecule[136]. As binding of the enzyme to the interface is a separate step from the catalytic reaction classical Michaelis-Menten kinetics may not apply. Issues regarding the involvement of a lipid-water interface in the catalytic process of lipolysis have been subject to an extensive review by Brockman[136], the main points of which are discussed in the following text.
In order for lipolysis to occur the substrate needs to be present at a concentration exceeding its solubility in the aqueous medium to create an interface. The interfacial area of substrate available for enzyme to bind is also relevant to the rate of lipolysis. This interfacial requirement of lipase may in fact be advantageous to enzyme activity as it helps to ensure enzyme and substrate accumulate together in high concentrations within a limited area for kinetic activity[137].

The velocity of a lipolytic reaction would be expected to relate to the ratio of enzyme to substrate interfacial area rather than substrate concentration in the bulk reaction mixture. If the substrate concentration is expressed in units of area / volume lipase activity is related to the interfacial area available. However the situation is complicated by partitioning of substrate from the bulk substrate phase into the interface which in turn relates concentration at the interface back to that present in the bulk phase.

The concentration of triglyceride available at the interface for lipolysis has been reported to be as low as 2 to 5 % with the amount decreasing with time[138]. However as lipolysis continues the lipid core of the emulsion droplets helps to maintain a rapid equilibrium between lipid in the bulk and surface phase to ensure replenishment of interfacial triglyceride. Diglycerides will accumulate at the interface in preference to triglyceride due to their higher surface activity conferred by the free hydroxyl group, however these will quickly be removed by rapid hydrolysis.

For the work carried out in this study weight / volume units were selected to express substrate concentration as opposed to units which define the interfacial area. This was due to the selection of an emulsion formulation for the substrate. Measurements of interfacial area for an emulsion system are difficult to determine[136] as are the lipid composition, lipid packing and surface pressure for such an interface[139].

The intended examination of the effect of surfactants on the kinetic parameters of pancreatin also argues against the use of interfacial area as a parameter. The addition of surfactant to a reaction system would be expected to cause an apparent increase in interfacial area due to incorporation of some surfactant molecules into the lipid interface. The parameter of interfacial area would therefore no longer solely represent the substrate concentration[136], hence comparison between kinetic parameters from substrates of MCT alone and in the presence of surfactant would not be possible.

3.1.2 Nature of the interface.

An interface which separates two bulk phases can be considered to be a two-dimensional surface known as the surface phase[136]. The surface phase will not be an homogeneous region and can coexist with other surface phases if more than two bulk

phases are present. Even a pure surface phase composed of a single component will not necessarily have all the molecules in the same conformation. When a surface phase has two or more components there are numerous modes of molecular interaction. The types of arrangements can range from immiscible to completely miscible, with changes in arrangement possible due to variations in the stoichiometry.

When the concept of surface phases is applied to a lipid-water interface it becomes evident that a lipid substrate presented as an emulsion will be composed of at least two distinct bulk phases[140] with surface phases between. The substrate and enzyme will partition between the bulk and surface phases along with digestion products produced as the substrate starts to undergo lipolysis. As the reaction proceeds substrate, enzyme and digestion products will undergo partitioning between different bulk and surface phases and their position will change with respect to time.

Factors which may affect partitioning include changes in interfacial tension, lipid packing arrangements, substrate concentration and area of interface available to lipase, all of which are altered due to the generation of lipolytic products. Lipase itself *in vitro* will not be present in high enough concentration to have a significant physical effect on phase distribution.

The arrangement or conformational state of the substrate at the surface phase will influence substrate availability for lipolysis[141] and hence affect the kinetic behaviour of lipase. Lipolysis may be limited to particular types of surface phases, have specific rates of activity for different surfaces or occur only at phase boundaries making reaction rates for lipolytic enzymes difficult to interpret.

3.1.3 Presentation of the substrate as an emulsion.

When investigating the kinetic activity of lipase the results will be influenced by the chosen experimental method as different techniques can alter physicochemical properties of the substrate and hence enzyme activity. For this work an indication of enzyme activity which could be related to the physiological situation was desired. Presentation of the triglyceride substrate as an emulsion allowed lipase to be in contact with substrate in a reaction mixture representative of *in vivo* conditions, as alimentary triglyceride upon ingestion forms a fat rich emulsion.

Under conditions of the standard pH-stat assay used in these kinetic studies the triglyceride is initially mechanically dispersed into simulated bile solution to produce a crude emulsion. Lecithin contained within simulated bile solution provides a degree of emulsification which is further enhanced by the digestion products released as lipolysis proceeds. The triglyceride therefore becomes increasingly emulsified over the course of the lipolysis reaction. This is similar to the *in vivo* situation where emulsion droplets have a core of non-polar lipids with a surface monolayer of amphiphiles. This monolayer usually consists of lipolytic products and phospholipids which create a mixed lipid film necessary to lower interfacial tension and prevent droplet aggregation. Bile salts are also present but these have little emulsification properties towards triglyceride[142].

In addition to the close representation of *in vivo* conditions given by use of an emulsion, a further advantage is the large interfacial area an emulsion provides. The interfacial area is the major factor which will influence the observed kinetic activity of lipase[143], regardless of different kinetic behaviour at various surface types. Providing sufficient interfacial area for all enzyme molecules to adsorb to the surface will result in determination of the maximal catalytic rate. The surfaces and stirrer of the reaction vessel also increase surface area available to lipase and may even enhance enzyme activity.

The special considerations necessary for kinetic studies involving lipase resulted in the following approach with regards to the methodology employed with this work. Due to the need for lipase to bind to a triglyceride interface before digestion can commence the triglyceride substrate was presented as an emulsion. This method of substrate presentation helps to maximize interfacial area available for lipase binding. The concentration of triglyceride present in each digestion performed was expressed in units of bulk concentration as these were easy to determine and not influenced by the presence of surfactant if included in the system.

3.1.4 Outline of this investigation.

With all the issues related to kinetic behaviour of lipase in mind a pragmatic approach was needed. It was decided in this study to investigate whether traditional enzyme kinetic equations could be applied to lipolysis of an emulsion system.
The study was designed to investigate the kinetic behaviour of pancreatin when digesting a MCT, (represented by Miglyol® 812), under conditions of the standard pH-stat assay. The behaviour of pancreatin was defined by determination of apparent values for the parameters, K_m and V_{max}. These parameters describe a hyperbolic relationship between initial velocity of an enzyme reaction and substrate concentration and are discussed further in section 3.2.
Inclusion of the surfactant Cremophor in the reaction system with MCT was known to inhibit pancreatin activity from preliminary studies. A further investigation was therefore designed to determine K_m and V_{max} values when Cremophor was included in the substrate emulsion. A knowledge of these apparent parameters was intended for further studies involving quantification of the potency of inhibition of various surfactants towards pancreatin.

The investigations carried out in this chapter are summarized below:

- Determination of K_m and V_{max} for pancreatin when acting upon MCT.
- An attempt to determine K_m and V_{max} for pancreatin when acting upon MCT in the presence of Cremophor. The experimental results were examined for information regarding the type of inhibition mediated by Cremophor.
- The effect of different batches of pancreatin on MCT hydrolysis in the presence of Cremophor.
- The effect on initial rate determinations of using 0.1 M NaOH as titrant in the standard pH-stat assay instead of the standard 1 M NaOH.
- The effect of the method of preparation used for the reaction mixture upon the rate of MCT hydrolysis.

3.1.5 Materials

As for Chapter 2 (section 2.2.1) plus

Cremophor RH40 (USP name-Polyoxyl 40 Hydrogenated Castor Oil)
Cremophor RH40 is a non-ionic surfactant described as a hydrogenated castor oil ethoxylate with a stated[144] average of 45 ethoxy groups per molecule and a HLB value of between 14 to 16. The general structure for a castor oil ethoxylate is illustrated in section 4.8.
Cremophor RH40 is produced by reacting 45 moles ethylene oxide with one mole of hydrogenated castor oil. This results in a composite product with glyceryl polyethylene glycol oxystearate and fatty acid glycerol polyglycol esters comprising the hydrophobic portion. The hydrophilic part consists of polyethylene glycol (PEG) and glycerol ethoxylate.

3.2 Determination of the kinetic parameters, K_m and V_{max} for pancreatin acting upon MCT.

3.2.1 Introduction

The kinetic parameters K_m and V_{max} are useful to describe the properties of an enzyme catalysed reaction. They describe the shape of a rectangular hyperbola predicted by the Michaelis-Menten equation[135] for a particular enzyme acting under specified reaction conditions.
The Michaelis-Menten equation (Equation 3.1) relates the rate of substrate utilization or product formation (v - hereafter referred to as velocity) of an enzyme reaction to the initial substrate concentration (s) present.

$$v = \frac{V_{max}\, s}{K_m + s} \qquad \text{Equation 3.1}$$

K_m and V_{max} are constants of this rectangular hyperbola relating velocity to substrate concentration. V_{max} is the maximum velocity of the reaction when the substrate

concentration is saturating the enzyme. K_m is the Michaelis constant describing the substrate concentration at which the reaction proceeds at half its maximum initial velocity. The following assumptions[145] are made during the derivation of the Michaelis-Menten equation:

- The initial rate measurement is made in the steady state when the concentration of enzyme-substrate complex is unchanging.
- The rate of the reverse reaction is negligible during the initial rate measurement period.
- The formation of enzyme-substrate complex does not significantly deplete the concentration of free substrate.

If these assumptions are valid then the initial portion of the progress curve representing the enzyme reaction will exhibit linear behaviour. The gradient of the linear portion is taken to be the initial velocity. This ensures that a steady-state has been reached when velocity is measured and any reverse reaction is negligible. To ensure that formation of enzyme / substrate complex does not deplete the substrate concentration a check should be made to ensure velocity is linearly proportional to enzyme concentration. Linearity between velocity and enzyme concentration for pancreatin activity upon MCT has already been discussed and demonstrated in Chapter 2.

The aim of this work was to characterize pancreatin activity, (represented by the initial velocity of the enzyme), towards a substrate of MCT, in terms of K_m and V_{max}. Progress curves of digestion of MCT by pancreatin (hereafter referred to as digestion profiles) were performed over a range of substrate concentrations to obtain initial velocity measurements. These provide a means of assessing the compliance of pancreatin behaviour with the Michaelis-Menten relationship.

The initial velocity of the enzyme reaction was determined under conditions of the standard pH-stat assay. This is a direct continuous assay which has the advantage of allowing observation of the rate of product formation, namely fatty acid, with respect to time. A curve is normally obtained with an initial linear rate which eventually declines with time. Any non-linear behaviour at the beginning of the reaction would be

apparent from visualization of the digestion profile reducing error in the estimation of initial velocity. Decreased enzyme activity due to a reduction in substrate availability, the reaction reaching equilibrium, product inhibition or instability of assay components would also be evident.

3.2.2 Method

A series of fourteen digestion profiles of pancreatin acting upon MCT, over a concentration range of 2 to 71 mM, were produced under conditions of the standard pH-stat assay. To increase the range of the hyperbolic plot of v against s, the substrate concentrations were chosen to span at least five times the K_m value.
The digestion profiles represented product formed, expressed in terms of fatty acid released in millimoles per unit volume, with respect to time. To obtain a measurement of initial velocity each digestion profile was inspected to ensure the initial portion was linear. The initial rate was then calculated from the gradient of the initial linear portion which normally occurred over the first five minutes from the start of digestion. Computerized linear regression was employed to calculate the gradient.

3.2.3 Calculation of K_m and V_{max}.

The initial velocity measurements obtained were used to produce a plot of v against v/s (Figure 3.1) known as an Eadie-Hofstee plot[146, 147]. This linear arrangement of the Michaelis-Menten equation best reveals fit upon visual inspection between the data and a straight line in order to check if the equation applies[148]. The plot also allows qualitative examination of the extent of scatter which relates to accuracy of the assay method (representation of error with this plot is distorted making quantitative assessment of error difficult[149]).
Upon visual inspection of the Eadie-Hofstee plot (Figure 3.1), a linear relationship was seen to exist ($r = -0.9593$) between substrate concentration and initial velocity indicating that the Michaelis-Menten relationship was applicable despite the heterogeneity of the reaction mixture.

There are several methods available for calculation of V_{max} and K_m values using the experimental initial velocity data obtained. With this work two methods were selected, the direct linear plot[150] and least-squares fit to a hyperbola[151], both of which can be calculated by a computerized procedure. These methods are preferred to methods involving linear transformations of the Michaelis-Menten equation which require the use of suitable weighting for each data point.

3.2.3.1 The direct linear plot.

The direct linear plot is created by plotting the substrate concentration used, *s*, and initial velocity value, *v*, obtained from each digestion profile as an individual pair. The *v* value is plotted onto the vertical axis representing V_{max} and the corresponding *s* value onto a negative horizontal K_m axis. A straight line is then drawn between the two points and extrapolated into 'V_{max}, K_m parameter space'.
The resulting straight lines obtained from each digestion profile should all intersect at the coordinates of the best fit K_m, V_{max} values if the data fits the Michaelis-Menten relationship exactly. Measuring errors present in experimental data result in different intersections being obtained, the coordinates of which each provide estimates of K_m and V_{max}. The median values of the intersection co-ordinates are taken to be the best fit K_m and V_{max}[152].
The advantages[145] of the direct linear plot include no requirement for a weighting scheme, the method is less sensitive to outliers than the least-squares fit method and confidence limits can be calculated for K_m and V_{max}. Only one assumption is made, that the error is as likely to be positive as negative.

Results

The *s* and *v* pairs obtained from the digestion profiles of pancreatin acting upon MCT were used to construct a direct linear plot (Figure 3.2) for illustration purposes. From Figure 3.2 a number of different intersections can be seen to occur due to measurement error in the data. A computer software package[153] was used to calculate the coordinates of each intersection and thus determine best-fit median values of K_m and V_{max}. The resulting K_m and V_{max} values are shown in Table 3.1.

Visual inspection of the direct linear plot will not reveal if the data significantly deviates from Equation 3.1, however a qualitative check for deviation had already been made by means of the Eadie-Hofstee plot (Figure 3.1).

3.2.3.2 Least-squares fit to a hyperbola.

This method involves a computerized least squares fit of a v against s hyperbola to experimental data points, resulting in best fit values of K_m and V_{max} and an estimate of their errors. To check assumptions made by the least squares fit method hundreds of points are necessary, however if justified the method gives marginally better estimates when compared with the more statistically robust direct linear plot.
The following assumptions[145] are implicit when using least squares fit method for the statistical analysis of enzyme kinetic data:

- Random errors in replicate values of the measured velocity, v, follow a normal distribution.
- There is no error in the substrate concentration, s.
- The correct weights are known.
- Fluctuations in the y values must be independent of any fluctuations in the x values.

Results

A curve fitting program[154] was used to fit the experimental data from the digestion profiles of pancreatin acting upon MCT to Equation 3.1. The resulting fit of the data points to a v against s hyperbola is shown in Figure 3.3.
The K_m and V_{max} values determined by the least squares fit (Table 3.1) are based on the presence of a constant absolute measurement error in the data. The existence of this homogenous error is an inherent assumption of the least squares fit method which is not necessarily true for the data.
The data may exhibit systematic variation of standard deviation which is dependent on the value of v, known as constant relative error. Allowance for this heterogeneous error would be need to be made by application of additional weighting factors to the

data. To do this a prohibitively large number of data points are required to confirm the correct weighting scheme.

Method of analysis	V_{max} (mmol min^{-1})	V_{max} statistics	K_m (mM)	K_m statistics
Direct linear	0.382	0.373 - 0.391 (68 % confidence limits)	11.6	10.6 - 12.5 (68 % confidence limits)
Least squares fit to hyperbola	0.375	0.008 (standard error)	10.8	0.77 (standard error)
Least squares fit to hyperbola with constant relative error	0.385		12.0	

Table 3.1 Comparison of parameter values K_m and V_{max} (for pancreatin acting upon MCT) determined using different analytical methods.

3.2.4 Discussion

As expected, the direct linear method and the least squares fit to a hyperbola gave different estimates for K_m and V_{max} when calculated from the same experimental data set, however they agreed to within confidence intervals so the difference was not significant. In order to analyze this result further another least squares fit was performed with weighting applied to the data which presumed existence of a constant relative error.

The application of a constant relative error gave values for K_m and V_{max} (Table 3.1) which, when compared with values from the other two methods, were closest to those given by the more statistically robust direct linear method. This gave an indication that the data exhibits heterogeneous error, describing an increase in measurement error as velocity value increases.

Parameter values calculated from the direct linear plot were therefore considered to be the most reliable as this method has no requirement for a weighting scheme. This was combined with the fact that to use the least squares fit method with confidence many more data points were necessary to confirm the correct weighting scheme.

Support for accuracy of the data overall and confidence in the parameter values obtained from the direct linear plot and the least square fit is given by Cleland[155]. Cleland stated that reasonably precise estimates of K_m and V_{max} would have standard deviations of less than 10 % of the mean K_m and V_{max} values. This was found to be true for both methods as in the case of the direct linear plot the standard deviation in terms of the percent of the mean is 8.19 % for K_m and 2.36 % for V_{max}, and the values from the least squares fit method are 7.16 % for K_m and 2.13 % for V_{max}.

The good fit of the experimental data to Equation 3.1 increased similarity of K_m and V_{max} values obtained via the two analytical methods. Therefore the choice of analytical method would probably not alter conclusions drawn from the use of K_m and V_{max} values in subsequent experiments.

3.2.5 Relevance of kinetic parameters to an enzyme which acts at an interface.

The *v* against *s* hyperbola (Figure 3.3) demonstrates the existence of a relationship between the initial velocity of the reaction and concentration of MCT present in the substrate emulsion. As substrate concentration is increased the enzyme eventually becomes saturated with substrate.

This result gives the impression that the Michaelis-Menten equation applies to the relationship between initial velocity of pancreatin and the concentration of MCT. However it has to be remembered that the Michaelis-Menten relationship is not directly applicable to the action of an enzyme upon an insoluble substrate. To explain the kinetics of a lipolytic enzyme acting at an interface, a model of two separate stages has been proposed resulting in a two-dimensional form of the Michaelis-Menten equation[156]. The first stage is an equilibrium to describe the reversible penetration of enzyme into the interface. Once penetration has occurred a second equilibrium exists for the catalytic interaction between a molecule of enzyme and a substrate molecule.

Various complications are involved in the interpretation of K_m or V_{max} determined in this study. The concentration of substrate was expressed as the total MCT present in the system. This resulted in a *v* against *s* hyperbola with *s* described as units of millimoles per unit volume. However the actual concentration of lipid available to the

enzyme may not directly relate to the total lipid present. The quantity of lipid partitioned from the bulk into the surface phase or the emulsion droplet size and hence interfacial area may vary, limiting availability of substrate for enzyme adsorption. If this is the case kinetic parameters determined will reflect the adsorption of lipase to the interface and interfacial interaction rather than solely the rate of catalytic interaction between substrate and enzyme.

It has been stated that when utilizing an emulsion form of substrate for determination of kinetic parameters all experiments should be performed using the same substrate preparation due to the non-equilibrium nature of emulsion particles and differences in particle size distribution[136]. Under conditions of the pH-stat assay it is difficult to know how relevant variations in emulsion droplet size would be once lipolysis commenced. The simulated bile solution along with lipolytic products released will aid triglyceride emulsification. This, combined with the mechanical stirring action of the pH-stat, could result in emulsion droplets of uniform size irrespective of substrate concentration thus the effect on the kinetic parameters determined could be minimal. To ensure the degree of substrate dispersion does not affect K_m or V_{max} substrate concentration could be expressed in terms of interfacial area, however as already discussed interfacial area is difficult to determine for an emulsion system, particularly one which is constantly changing.

Regardless of the factor/s controlling the increase in enzyme velocity with substrate concentration, the relationship between the two appears to be of a consistent manner for pancreatin activity upon MCT under conditions of the standard pH-stat assay. This gives support to the determined values of K_m and V_{max} for subsequent comparison with the same parameters measured in the presence of surfactant.

3.3 Investigation of the inhibition of pancreatin activity by the surfactant, Cremophor.

3.3.1 Introduction

Knowledge of the mechanism by which surfactants inhibit pancreatin activity may be useful to prevent inclusion of surfactants with this property in lipid-based

formulations. Preliminary experiments by colleagues at R P Scherer[108] had revealed pancreatin activity to decrease towards a substrate of MCT in the presence of the surfactant, Cremophor. As the kinetic behaviour of pancreatin upon MCT (section 3.2) exhibited apparent compliance with the Michaelis Menten equation an investigation was designed to examine whether this compliance still held when Cremophor was included in the system. If this was the case elucidation of the type and mechanism of inhibition shown by Cremophor towards pancreatin may be possible.

The use of the term inhibitor describes any substance which can reduce the velocity of an enzyme-catalysed reaction. Studies of the inhibition of enzyme activity can be useful to provide information regarding the specificity of an enzyme, the active site of an enzyme and / or kinetic mechanisms involved in the reaction[117].
Inhibitors can be divided into two main types, irreversible and reversible[151].
Irreversible inhibitors combine with the enzyme to cause eventual zero catalytic activity if present in excess to the enzyme concentration. Enzyme activity cannot be regenerated by removal of an irreversible inhibitor from the system. Irreversible inhibition is quantified in terms of the velocity of inhibition.
Reversible inhibitors exist in an equilibrium between free and bound state with the enzyme or enzyme-substrate complex. The effectiveness of a reversible inhibitor is expressed by the inhibitor constant (k_i)[109], which is a quantitative measure of the dissociation constant for the enzyme-inhibitor complex. k_i can be useful to compare potency of different inhibitors when acting under specified conditions upon the same substrate.
Reversible inhibitors can be classified into three main types, competitive, non-competitive and uncompetitive with partial inhibitors existing for each type[109]. Mixed inhibitors which exhibit a combination of the above types also exist. The types are classified according to the step in the catalytic pathway where the inhibitor acts. Distinction between the different types of inhibition can be achieved by graphical analysis although a definite classification on this basis alone is not always possible. A linear arrangement of the Michaelis Menten equation, the Lineweaver-Burk double reciprocal plot[157] ($1/v$ against $1/[S]$) is normally chosen for this purpose. The effect of the presence of inhibitor on the plot of $1/v$ against $1/[S]$ can also be used for the

determination of k_i, however it is preferable to perform a least squares fit to the appropriate rate equation if known.

To classify the type of inhibition using the Lineweaver-Burk method, data needs to be collected for construction of several line plots of $1/v$ against 1/[S], with each plot made in the presence of a different fixed concentration of inhibitor[158]. All of the plots are then assembled on one set of axes. Determination of the type of inhibition is made on the basis of how the presence of the inhibitor effects the relationship between the line plots and the position of their intersections with relation to the axes.

If an inhibitor bound to an enzyme causes complete inhibition of a reversible nature the plot of $1/v$ against 1/[S] will be sufficient to identify the type of inhibition. Further information on partial inhibition may be revealed when gradients taken from the Lineweaver-Burk plot are shown against the fixed inhibitor concentration used[109]. For example in the presence of a partial inhibitor an enzyme will retain some catalytic activity when the inhibitor is bound. Gradients from the Lineweaver-Burk plot against inhibitor concentration will show hyperbolic behaviour indicating a partial inhibitor rather than the usual linear relationship seen with a complete inhibitor.

Further information on the type of inhibition can be gained by comparison of the kinetic parameters K_m and V_{max} obtained in the absence of an inhibitor, with those given by the identical enzyme reaction when inhibitor is present[151]. Competitive inhibitors increase the value of K_m, which means that a higher substrate concentration is necessary to achieve half the velocity of V_{max} which remains constant. Non-competitive inhibitors reduce V_{max}, the maximum velocity attainable, while K_m is unchanged. Uncompetitive inhibitors decrease both K_m and V_{max} whilst mixed inhibitors can increase or decrease the value of either parameter.

The purpose of this work was to use the Lineweaver-Burk method to classify the type of inhibition displayed by Cremophor towards pancreatin activity upon MCT. In addition information regarding the site of inhibitory activity may become apparent. Quantification of the potency of inhibition of Cremophor towards pancreatin measured by the inhibitor constant, k_i may also be possible.

3.3.2 Method

Digestion profiles were produced to obtain five Lineweaver-Burk plots of $1/v$ against 1/[S], with each plot produced in the presence of a different fixed concentration of Cremophor. Concentrations of MCT substrate (0.1 g, 0.25 g, 0.5 g, 0.75 g, 1 g, 1.5 g, 2 g, 3 g and 4 g) for each Lineweaver-Burk plot were selected using Figure 3.3 as a guideline. A preliminary investigation of Cremophor inhibition of pancreatin activity towards MCT was performed (results not shown) to ensure the selected fixed Cremophor concentrations (0.01 g, 0.05 g, 0.1 g, 0.25 g and 0.5 g) gave varying degrees of inhibition.

A digestion profile was produced for each MCT concentration in the presence of each of the five Cremophor concentrations in turn. This resulted in the initial production of 45 digestion profiles with some combinations being replicated later. Profiles were produced under conditions of the standard pH-stat assay with the titrant volume recorded every minute for a total period of twenty minutes.

3.3.3 Results

Determination of initial velocity.

To construct the $1/v$ against 1/[S] plots initial velocity measurements were required from each digestion profile. A visual inspection of the digestion profiles revealed that some profiles were exhibiting a lag phase. A lag phase is a term used to describe a slowly accelerating phase of enzyme activity which occurs at the beginning of the progress curve[132]. The lag phase is then followed by a phase of higher enzyme activity where the enzyme has reached the linear steady state. With the presence of a lag and steady state phase in the same digestion profile pancreatin can be said to be demonstrating biphasic kinetics[109]. Reasons for the appearance of a lag phase are discussed later in the text (section 3.3.6).

The presence of a lag phase introduced complications in relation to measurement of initial velocity. Some differences in the nature of the digestion profiles obtained in the presence of Cremophor are illustrated in Figure 3.4. For digestion profiles showing a definite lag and steady state phase followed by an approach towards equilibrium, (e.g.

profile B, Figure 3.4), the initial rate can be determined from the steady state phase with reasonable accuracy. On some digestion profiles (e.g. profile D, Figure 3.4) the position where the lag phase finished and the steady state commenced was difficult to assess. In these cases the series of linear data points which exhibited the steepest gradient were used to calculate maximum enzyme velocity shown on the digestion profile.

The duration of the assay period was a further complication as a higher rate of enzyme activity may have been reached later in the profile if the assay had been continued for a longer period (e.g. profile C, Figure 3.4). Digestion profiles produced in the presence of high concentrations of Cremophor (e.g. 0.5 g) exhibited extensive suppression of enzyme activity (e.g. profile E, Figure 3.4). These profiles were suspected to be composed solely of a lag phase which had not been overcome within the 20 minute assay. In view of the linear nature of profiles of type E the initial velocity measurements were taken to be the gradient of the total profile.

For all digestion profiles the initial velocity measurements were calculated using linear regression on the selected titrant volumes. An attempt to overcome difficulties in initial rate determination due to presence of a lag phase could have been made by fitting each digestion profile to a rate equation for a given type of inhibition. However as the inhibition mechanism was not known and could not be hypothesized this was not possible.

The Lineweaver-Burk plot.

The initial velocity measurements determined were presented as individual Lineweaver-Burk plots of $1/v$ against 1/[S] for each fixed concentration of Cremophor (Figures 3.5 a-e). These plots (Figures 3.5 a-e) may be exhibiting a linear relationship between $1/v$ and 1/[S] although the scatter was too great to make a definite statement to this effect.

A criticism of the Lineweaver-Burk plot is that it gives undue weighting to the points obtained at low substrate concentrations (high 1/[S] values)[158]. This can result in concealment of a poor fit between data and a straight line if proper weighting is not applied to the points[109]. To illustrate this error bars for v of $\pm$ 0.01 mmol min^{-1} have been applied to the experimental data in Figure 3.5a to show the deficiencies of the

plot. This is only a rough estimate of the error, not an accurate quantitative assessment of the experimental error. The positive error bars for high values of 1/[S] are not shown as they exceeded the scale of the graph.

Although the Lineweaver-Burk plot is not reliable for calculation of kinetic parameters, the plot was selected for this work to enable examination of trends due to the presence of an inhibitor. In view of the manner in which error is increased at high 1/[S] values this needs to be born in mind with regards to assessing linearity of the data. Further examination of the data in Figures 5.3 a-e when represented as separate v against s hyperbolas suggested the presence of Cremophor had altered the behaviour of pancreatin in such a manner that the enzyme kinetics were no longer related to the Michaelis-Menten equation.

Figures 3.5 a-e were combined onto a single Lineweaver-Burk plot (Figure 3.6) to enable examination of the relationship between data sets obtained using different Cremophor concentrations. The resulting combined Lineweaver-Burk plot (Figure 3.6) did not exhibit a series of straight line plots which could be discussed with any confidence to provide information on the type of inhibition shown by Cremophor. Any relationship between the data sets was difficult to identify due to the large degree of scatter in the data.

There are several experimental reasons which may have caused pancreatin activity in Figure 3.6 to exhibit non-linear kinetics when in fact the enzyme may still be obeying the Michaelis-Menten equation. The contribution of the lag phase to error in initial velocity measurements has already been discussed. For some profiles initial velocity may not have been determined at steady state if the lag phase had not been overcome within the duration of the assay. Pancreatin has shown the ability to overcome inhibition from Cremophor with time (Figure 3.12c). In retrospect extension of the duration of the assay would probably have eliminated this source of error, by allowing measurement of the steady state reaction rate on profiles displaying a high degree of inhibition.

Another experimental influence which may have prevented pancreatin activity from exhibiting linear kinetics was the use of different batches of pancreatin for the digestion experiments. This situation was enforced by the supply of materials. An investigation into effects from the use of different batches of pancreatin is described in

section 3.4. Although experimental error could have lead to scatter in Figure 3.6, the overall effect was felt unlikely to be sufficient to obscure an overall trend between the line plots if one existed.

3.3.4 Discussion

The inhibitory effect of Cremophor upon pancreatin activity could not be classified in terms of a particular type of inhibition using the Lineweaver-Burk method. This was due to non-compliance of pancreatin with the Michaelis-Menten equation when Cremophor was present or to experimental error in the results as already discussed. The manner in which Cremophor may be able to inhibit pancreatin is therefore discussed qualitatively below in terms of the three main types of inhibition[117]:

- Competitive inhibition

A competitive inhibitor competes with substrate for the same binding site upon free enzyme. Alternatively the inhibitor may bind at a different site to the substrate on the enzyme and cause a conformational change in the enzyme so that substrate can no longer bind to its normal binding site. This means the inhibitor would not necessarily have to resemble the substrate structure to display competitive kinetics.

The extent of competitive inhibition can be reduced by increasing the substrate concentration whilst inhibitor concentration remains fixed. If an increase in substrate concentration is able to reduce inhibition an increase in the K_m value will be observed whilst V_{max} remains the same when compared to kinetic parameters obtained in the absence of inhibitor.

There is evidence that non-ionic surfactants can inhibit the activity of lipase by binding in the active site of the enzyme. Crystallographic studies of porcine lipase and colipase with the non-ionic surfactant, tetraethylene glycol monooctyl ether (TGME) revealed a TGME molecule to be tightly bound to the active site pocket[159]. The non-ionic surfactant in this instance probably induced opening of the active site flap acting as a substrate analog. Further support for this is given by the observed reversible inhibition of tributyrin hydrolysis when TGME was included in the system.

Cremophor could also act as a competitive inhibitor by binding at the active site on lipase in the same manner as TGME or by interfering with the interfacial binding site on lipase or colipase; this may result in a conformational change of lipase or colipase with derogatory effects on enzyme activity.

Evidence to suggest that Cremophor acts as a competitive inhibitor would be the demonstration of identical values of V_{max} when acting upon MCT alone and in the presence of Cremophor. This should be achieved solely by means of increasing the substrate concentration. In the absence of Cremophor V_{max} is reached at a MCT concentration of 3.5 g. Unfortunately due to limitations of the assay system it was not possible to increase MCT concentrations to the higher levels necessary to reach V_{max} in the presence of Cremophor.

- Non-competitive

A non-competitive inhibitor forms an inactive enzyme /substrate / inhibitor complex by binding at a site on the enzyme other than the substrate binding site. The inhibitor is also able to bind with the free enzyme whilst not altering binding of enzyme to substrate. For Cremophor to exhibit non-competitive inhibition an inactive complex of lipase or colipase with MCT and Cremophor would need to be formed. The resulting complex could not break down to release product or would break down at a slower rate than lipase with MCT if exhibiting partial non-competitive behaviour.

The effect of a non-competitive inhibitor cannot be overcome by increasing the substrate concentration as a proportion of enzyme remains in the inactive complex. As a result at a fixed concentration of inhibitor, the velocity of the reaction will be reduced by the same degree of inhibition, independent of the substrate concentration used. The effect of this on kinetic parameters is a decrease in V_{max} whilst the K_m value remains constant compared to the value in the absence of inhibitor.

To examine for changes in kinetic parameters due to the presence of Cremophor the experimental data obtained using 0.01 g of Cremophor (Figure 3.5 a) was selected. This data was chosen as when plotted as a *v* against *s* hyperbola (Figure 3.7) it displayed the least scatter when compared with the other Cremophor concentrations tested (Figure 3.5 b-e). The experimental data (Figure 3.7) was fitted to Equation 3.1 via a computerized least squares fit of a *v* against *s* hyperbola to obtain estimates for

K_m and V_{max} (Table 3.2). The least squares fit method was chosen instead of the direct linear plot as it allows visual examination of the deviation from Equation 3.1. Although the parameters obtained exhibited a high degree of error they provided a qualitative indication that the presence of Cremophor decreased V_{max} by approximately one third compared to V_{max} for MCT alone (Table 3.1), whilst the value of K_m increased threefold compared to the K_m for MCT alone (Table 3.1). The increase in the K_m value tends to suggest that the mechanism of inhibition shown by Cremophor is not non-competitive.

- Uncompetitive

An uncompetitive inhibitor is unable to bind with free enzyme and can only bind reversibly with the enzyme / substrate complex causing it to become inactive. As the inhibitor is only able to bind with the enzyme / substrate complex the degree of inhibition increases in line with substrate concentration. This mechanism is considered rare in single substrate reactions and results in a decrease in V_{max} and K_m compared to the same parameters given in the absence of inhibitor.
For Cremophor to exhibit uncompetitive inhibition it would only be able to combine with a lipase / MCT complex probably with colipase also present and would not be able to bind with free lipase. From examination of K_m and V_{max} values (Table 3.2) obtained using 0.01 g of Cremophor an uncompetitive mechanism seems unlikely as K_m increased compared to the value given in the absence of inhibitor.
Mixed inhibitors also exist which have an effect on both V_{max} and K_m. These can arise from several situations therefore it is not possible to give an explanation of the possible mechanisms involved.

From the above discussion, comparison of the kinetic parameters K_m and V_{max} for pancreatin activity upon MCT (Table 3.1) with those obtained in the presence Cremophor (Table 3.2) has indicated that non-competitive and uncompetitive mechanisms are unlikely. The definite increase seen in the K_m value corresponds to the expected change in kinetic parameters for competitive inhibition. The V_{max} value should be unchanged for a competitive inhibitor whereas with these results the V_{max} value is decreased. A decreased V_{max} value is to be expected as substrate

concentrations assayed in the presence of Cremophor were not increased above the levels tested for MCT alone. Without testing higher concentrations of substrate it is not possible to state that the maximum velocity had been reached. Therefore by comparison of the kinetic parameters K_m and V_{max}, Cremophor may be acting by way of a competitive mechanism or be exhibiting a mixed type of inhibition.

Degree of inhibition.

A distinction between the three main types of inhibition can be made by examination of a measurement known as the degree of inhibition[117] over a range of substrate concentrations. The degree of inhibition relates initial velocity measurements from the same concentration of substrate in the presence and absence of an inhibitor.

A selection of digestion profiles of pancreatin activity towards MCT in the presence of Cremophor (0.01 g) are shown in Figure 3.8. The inhibitory effect of Cremophor on pancreatin activity towards MCT appears to diminish when the substrate concentration is increased. Figure 3.8 illustrates the monotonic increase in initial velocity with substrate concentration indicating increased enzyme activity. This does not necessarily imply that inhibition is being overcome as the increase in velocity may solely be due to the presence of an increased concentration of substrate.

The degree of inhibition enables enzyme activity to be examined in relation to substrate concentration and the inhibitory effect of Cremophor. Equation 3.2 expresses the degree of inhibition as a percentage of uninhibited initial velocity, $V_{\%}$.

$$V_{\%} = \frac{V_i}{V_o} \times 100 \qquad \text{Equation 3.2}$$

where

V_i = Initial velocity measured in the presence of inhibitor for fixed concentration of substrate.

V_o = Initial velocity measured in absence of inhibitor for the same fixed concentration of substrate.

Equation 3.2 was used to calculate $V_{\%}$ for the initial velocity measurements in the combined Lineweaver-Burk plot (Figure 3.6). This was possible only for substrate concentrations where a corresponding uninhibited velocity measurement for MCT had been made in section 3.2. The resulting variation in $V_{\%}$ with substrate concentration is shown in Figures 3.9 a-b where the data is grouped according to the concentration of Cremophor used.

To gain information regarding the type of inhibition shown by Cremophor from Figures 3.9 a-b a trend should be seen for each Cremophor concentration across the range of substrate concentrations. For a competitive inhibitor the degree of inhibition (expressed as $V_{\%}$) should decrease as substrate concentration is increased, for a non-competitive inhibitor the $V_{\%}$ should remain constant whereas for a uncompetitive inhibitor $V_{\%}$ will increase with substrate concentration[117].

A trend was not evident between $V_{\%}$ and substrate concentration (Figures 3.9 a-b), due to the large degree of scatter in the results. It was therefore not possible to gain any further information regarding the type of inhibition shown by Cremophor using the parameter of the degree of inhibition. However all values of $V_{\%}$ were at or below 80 % indicating that in all cases Cremophor diminished the rate of enzyme activity in the steady state phase compared to the rate obtained using the same substrate concentration in the absence of Cremophor.

3.3.5 Conclusions

From this investigation into the inhibition of pancreatin activity by Cremophor it has not been possible to identify a particular type of inhibition. Results do however indicate that Cremophor is acting as a reversible rather than irreversible inhibitor. Evidence for this is supplied by comparison of digestion profiles for pancreatin acting upon MCT in the presence and absence of Cremophor. The presence of an identifiable lag phase on the digestion profile only occurs when Cremophor is included in the system.

Cremophor therefore appears to be responsible for inhibiting pancreatin activity in the early part of the digestion profile. As the lag phase is followed by a period of increased enzyme activity this indicates that inhibition is of a reversible nature. If the

inhibitor were irreversible enzyme activity would be completely lost upon binding with an inhibitor and the velocity would not be able to increase later on in the reaction.
A relationship between the concentration of inhibitor present and the extent of pancreatin inhibition at a fixed concentration of substrate has also been revealed. The effect of an increase in Cremophor concentration on digestion profiles obtained using a fixed substrate concentration (1 g MCT) is illustrated in Figure 3.10. The digestion profiles show a decrease in enzyme activity as concentration of inhibitor in the reaction mixture is raised.
To quantify this decrease in pancreatin activity the time taken to digest 2.5 % of MCT was calculated from each digestion profile. The resulting times are shown to increase with inhibitor concentration in Figure 3.11. For 2.5 % of MCT to be digested in the presence of 0.01 g of Cremophor takes 2 minutes whilst when Cremophor is raised to 0.5 g the time is increased to 14 minutes.
In conclusion the only statements it is possible to make from this investigation is that Cremophor acts as a reversible inhibitor towards the digestion of MCT by pancreatin. Enzyme activity in the steady state phase was reduced by Cremophor in all cases compared to the rate when Cremophor was absent. Furthermore the extent to which enzyme activity is inhibited for a fixed substrate concentration increases with the amount of Cremophor present over the range of Cremophor concentrations tested.

3.3.6 Significance of the lag phase in relation to the mechanism of Cremophor inhibition.

The lag phase observed on digestion profiles of MCT in the presence of Cremophor may be a result of the assay method or be a feature of the enzyme-substrate reaction [132]. As the digestion profiles of pancreatin activity towards MCT alone did not show a measurable lag phase this argues against the cause being an artefact of the assay system.
The presence of Cremophor in the assay altered the nature of the majority of digestion profiles obtained, with the resultant curve indicating biphasic reaction kinetics. In general the duration of the lag phase increased with the proportion of Cremophor to

substrate present. Lag phases were observed which ranged from a minimum of one minute to a maximum of the total assay period of twenty minutes.

Lag phases in kinetic studies of lipolytic enzymes acting upon monolayers, micelles and emulsions have been a common observation[131]. A lag time, τ_l, the duration of which was found to be independent of enzyme concentration, has been described for pancreatic phospholipase A acting upon phospholipid systems of low lipid packing[160]. τ_l was said to represent the rate limiting step of diffusion-controlled enzyme adsorption into the interface before a steady state was reached.

When the above observation is related to the studies performed here for pancreatin acting upon an emulsion system of MCT no observable lag time was present. The lack of a lag phase indicates that any delay in enzyme penetration into the interface of the triglyceride droplets is of a transitory nature and if present cannot be detected by the standard pH-stat assay. This may be due to the chosen conditions of the standard pH-stat assay in terms of pH, ionic strength and calcium ion concentration, all of which have been related to duration of the lag phase[139, 160].

The presence of a lag phase when Cremophor is included in the system suggests that Cremophor may have become incorporated into the interface of the triglyceride droplets or be present upon the interfacial surface. This could delay lipase or colipase adsorption to the interface to such an extent that the prolonged adsorption step is revealed by the assay as a lag phase.

Product activation is a common interpretation given to explain the lag phase seen when lipolytic enzymes act at a lipid / water interface[131]. This term describes the situation where products released from the lipolytic reaction such as fatty acids increase enzyme activity. The release of lipolytic products may increase enzyme activity by acting as amphiphiles to enhance substrate dispersion, or by increasing the substrate enzyme interaction through charge effects.

A further lag type, τ_d[139] has been described as dependent in duration upon the rate of product accumulation at the lipid / water interface and enzyme concentration. τ_d was observed from monolayer studies under conditions of high surface pressure which in turn relates to high lipid packing around triglyceride droplets. The duration of τ_d was proposed to be dependent on the effect of product accumulation at the interface which subsequently alters colipase partitioning.

The following theory of colipase partitioning[139] relates product accumulation at the interface to increased interfacial colipase binding and hence an increased lipolytic rate. Colipase can partition between the bulk aqueous phase and the lipid / water interface. Before initiation of lipolysis the colipase partition coefficient will be constant for a fixed interfacial composition, with colipase preferentially partitioned into the aqueous bulk phase. As lipolysis proceeds the interfacial composition will change as lipolytic products start to accumulate and colipase partitioning into the lipid / water interface will increase. Fatty acids released by lipolysis have been suggested[161, 162] to form clusters of between 10 to 20 molecules which become stabilized by calcium ions to form fatty acid-calcium soaps at the interface. These clusters could change the charge properties of the surface and enhance colipase binding and hence hydrolytic rate. Further support to this theory was provided by inclusion of calcium ions and albumin in the system[139]. An increase in calcium ions which encourage fatty acid cluster formation decreased duration of τ_d, whilst addition of albumin, known to cause desorption of products from the interface, increased duration of τ_d.

Although the work described above was carried out using monolayer techniques biphasic kinetics have also been reported from *in vitro* studies using substrate in emulsion form[163]. The emulsion consisted of dietary LCT droplets surrounded by a phospholipid monolayer, the packing density of which creates a high surface pressure. The above situation is comparable to the *in vitro* studies performed with this work using the standard pH-stat assay where biphasic kinetics are seen when Cremophor is included in an emulsion of triglyceride and phospholipid.

The appearance of a lag phase in pancreatin activity when Cremophor is included with MCT may be explained by a combination of mechanisms involving diffusion-controlled enzyme adsorption into the interface and product activation. The lack of a measurable lag phase on digestion profiles of MCT alone indicate that under the conditions of the standard pH-stat assay colipase can rapidly adsorb to the triglyceride interface for fast initiation of hydrolysis. When Cremophor is added to the system the appearance of a lag phase could indicate Cremophor molecules incorporated into or layered on the interface are restricting adsorption of colipase. As some colipase eventually becomes bound to the interface the mechanism of product activation may start to play a part. The released lipolytic products would accumulate at the

triglyceride interface perhaps replacing some of the Cremophor molecules whilst at the same time enhancing colipase partitioning. The overall effect would be a sudden increase in hydrolytic rate leading to the biphasic kinetics observed.
This hypothesis would also explain why the duration of the lag phase was not always consistent for digestion profiles produced using identical concentrations of Cremophor and substrate. A particular combination or concentration of products at the interface can have a dramatic effect on colipase partitioning. This can produce a sudden large increase in the hydrolytic rate resulting in lack of a linear relationship between colipase binding and lipolytic product concentration[139]. The transitory nature of fatty acid clusters altering charge surface properties at the interface may also help to account for the erratic lag duration apparent between digestion profiles of comparable substrate and inhibitor concentration. An investigation of the duration of the lag phase in relation to enzyme, fatty acid and calcium ion concentrations may be a useful area to research for further insight into the inhibitory mechanism of Cremophor.

3.4 Investigation of the effect of different batches of pancreatin on the attempt to classify the type of inhibition shown by Cremophor.

3.4.1 Introduction

Whilst investigating the inhibition of pancreatin activity due to the presence of Cremophor it became necessary to replenish the stock of pancreatin. Upon receipt the new supply of pancreatin (batch II) was noted to be of a different batch number to that already in use (batch I). The new supply was assessed for activity towards tributyrin (section 2.3) which indicated comparable activity to the previous batch I therefore work continued using batch II.
Analysis of results from later work in Chapter 4 revealed enzyme activity towards identical MCT / surfactant mixtures to vary when a different batch of pancreatin was used for the digestion. Batch I of pancreatin exhibited higher activity than batch II. Both batch I and II of pancreatin had been employed in the study of Cremophor inhibition of pancreatin activity towards MCT. As results obtained using both batches

had been analyzed together it was felt that the use of different enzyme batches may have been a contributory factor towards the large degree of scatter in the results obtained. A study was therefore designed to examine the inherent enzyme activity of each batch in terms of activity towards MCT / Cremophor mixtures.

3.4.2 Method

A series of experiments were designed to enable comparison between the different batches of pancreatin in terms of the extent of activity shown towards MCT / Cremophor mixtures. The batches of pancreatin to be tested were Sigma batch I, Sigma batch II (obtained 10/95), Sigma batch IIa (obtained 6/96) and a source of pancreatin from BDH (batch IV). Batch IV pancreatin was chosen as an example of an enzyme extract with stated higher minimum activity than the Sigma source.
The batch IIa pancreatin represented a fresh sample from the same lot number as batch II and was used in these experiments immediately upon receipt for comparison with results obtained from the original supply of batch II. This was to ensure no loss of activity had occurred to the original supply of batch II during use and storage in the laboratory. Further supplies of batch I pancreatin for this study were unavailable from Sigma, therefore as stock was exhausted data from digestions performed in section 3.3 were included for comparison when appropriate.
The investigation involved the digestion of MCT / Cremophor mixtures of varying proportions. The concentration of MCT was fixed at 1 g with the proportion of Cremophor ranging through 0.01 g, 0.05 g, 0.1 g, 0.25 g, 0.5 g and 1 g. Each combination was digested under conditions of the standard pH-stat assay using the batches of pancreatin available.

3.4.3 Results and discussion.

Digestion profiles of pancreatin activity towards the MCT / Cremophor mixtures were grouped in terms of the Cremophor concentration present. The digestion profiles illustrated the extent of digestion with time for the different batches of pancreatin tested (Figures 3.12 a-g). In general the results showed little difference in activity

between batch II and batch IIa of pancreatin however batch I appeared to have higher activity towards the substrate than batch II in most cases. Batch IV pancreatin exhibited higher activity than the Sigma source. This was to be expected from the slightly higher quoted minimum standard of activity compared to that stated for the Sigma source. The extent of the increase in batch IV pancreatin activity compared to Sigma pancreatin decreased with the concentration of Cremophor present. Overall the digestion profiles in Figures 3.12 a-g did not show a great difference in activity between batches of Sigma pancreatin and could not give weight to a hypothesis that batch I was more active towards MCT / Cremophor mixtures than batch II.

This investigation had only covered a limited range of the concentrations of MCT and Cremophor used for the inhibition study involving Cremophor in section 3.3. An examination was therefore made of the initial velocity measurements displayed in the combined Lineweaver-Burk plot (Figure 3.6) in terms of the batch of pancreatin used. It was thought that these results may reveal further information regarding variability between the batches of pancreatin which was not apparent when assessing the digestion profiles of the selected combinations tested above.

To reexamine results from Figure 3.6 in terms of variation in activity between batches of pancreatin the initial velocity measurements were separated according to the batch of pancreatin used for the digestion. The data from each batch is represented as a hyperbolic plot of v against s, using separate plots for each fixed Cremophor concentration (Figure 3.13a {batch I} and Figure 3.13b {batch II}).

The separation of results into batch of pancreatin used (Figures 3.13 a-b) indicated an apparent reduction of scatter within the batch II results compared to those given by batch I. This suggests that batch II is more likely to give reproducible results than batch I when acting upon MCT and Cremophor mixtures. However the apparent reduction in the degree of scatter could be incidental as Figure 3.13 a and b do not represent the same number of data points for each Cremophor concentration.

In conclusion the large degree of scatter within the total results from the Cremophor inhibition study shown in Figure 3.6 was felt to be due mainly to the lag phase as already discussed, with a small influence from variation in activity between batches of pancreatin due to the crude nature of the enzyme extract.

The digestion profiles obtained from the experiments performed in this study were replicates of MCT and Cremophor combinations already digested for the investigation of the inhibitory behaviour of Cremophor (section 3.3). After treatment of the digestion profiles as described in section 3.3.3 the initial velocity measurements obtained were included on the combined Lineweaver-Burk plot (Figure 3.6); digestion profiles produced using batch IIa and IV of pancreatin were excluded.
Variation in enzyme activity between batches of pancreatin was not an issue for the experimental determination of the kinetic parameters K_m and V_{max} (section 3.2) as batch I was used to produce all of the digestion profiles.

3.5 An investigation of the effect on initial rate measurements of using 0.1 M NaOH as titrant in the standard pH-stat assay.

3.5.1 Introduction

Throughout the study of inhibition of pancreatin activity due to Cremophor, a titrant concentration of 1 M NaOH had been used under conditions of the standard pH-stat assay to record the rate of enzyme activity. This provided fast neutralization of the protons released from fatty acids during the hydrolysis of triglyceride with minimal increase in volume of the reaction mixture over the duration of the assay.
The use of a high concentration of titrant may decrease assay sensitivity in terms of volume of titrant recorded with time. Addition of the lowest volume it is possible for the pH-stat to deliver could result in overshoot of the end point pH if reaction rate was sufficiently slow, although this had not been observed to occur from the pH of the reaction mixture displayed throughout each digestion. However the increased pH may have been rapidly compensated for by release of further protons from subsequent hydrolysis of triglyceride. The amount of NaOH added would therefore be correct but detail in the rate of titrant addition with time and hence the rate of reaction may be lost. The recorded titrant volume used to calculate initial velocity could therefore include titrant related to digestion occurring slightly later in the reaction.
An investigation was designed to examine whether a reduction in the concentration of titrant used could reduce scatter in the initial velocity values when fitted to Equation

3.1. This involved the production of a series of digestion profiles using 0.1 M NaOH as titrant. Initial velocity values obtained from these profiles and from replicate digestion profiles produced using 1 M NaOH as titrant were compared for compliance with the Michaelis-Menten equation.

3.5.2 Method

A series of digestion profiles representing pancreatin activity towards MCT in the presence of Cremophor were produced under conditions of the standard pH-stat assay. The Cremophor concentration was fixed at 0.01 g with substrate (MCT) concentration varying from 0.1 g to 2 g. The titrant used for the assay was a standardized solution of 0.1 M NaOH. The speed setting on the ABU 80 autoburette was increased to a capacity of 20 ml per minute to reduce any delay in reaction neutralization due to the increased volume of titrant used. All other settings remained as stated in section 2.2.2.

3.5.3 Results and discussion.

A lag phase was evident at the beginning of each digestion profile therefore initial velocity was calculated from the steepest gradient exhibited on the profile (as per section 3.3.3). Figure 3.14 illustrates the hyperbolic nature of the initial velocity values in relation to substrate concentration. A computerized least squares fit of a *v* against *s* hyperbola to the experimental data points (Figure 3.14) illustrates the apparent compliance of the data with Equation 3.1. This procedure also provided best fit values of K_m and V_{max} and an estimate of their errors, shown in Table 3.2.

Titrant concentration	V_{max} (mmol min^{-1})	V_{max} standard error	K_m (mM)	K_m standard error
1 0 M NaOH	0.226	0.031	32.4	8.83
0.1 M NaOH	0.184	0.016	21.7	3.83

Table 3.2 Comparison of K_m and V_{max} values for pancreatin when acting upon MCT (in the presence of 0.01 g of Cremophor), determined using different concentrations of titrant.

A reduction in titrant concentration from 1 M (Figure 3.7) to 0.1 M NaOH (Figure 3.14) appears to have increased compliance of pancreatin behaviour with the Michaelis-Menten equation. This is shown by comparison of the standard errors for corresponding values of K_m and V_{max} (Table 3.2). The larger titrant volumes when using 0.1 M NaOH may have increased precision when determining the initial velocity by improving identification of the steady state phase.

The use of a low Cremophor concentration of 0.01 g has already been shown to exhibit closer compliance to the Michaelis-Menten equation compared to results using higher concentrations of Cremophor (Figure 3.5 a-e), probably due to the reduced degree of inhibition of pancreatin activity. It is debatable whether the use of 0.1 M NaOH as titrant would have increased compliance of pancreatin behaviour with the Michaelis-Menten equation for digestion profiles containing concentrations of Cremophor above 0.01 g. Although this was not tested higher Cremophor concentrations tended to cause greater distortion in the shape of digestion profiles which would not be altered by a higher titrant volume therefore identification of the steady state phase would still be subjective leading to error. Any deviation in pancreatin behaviour from the Michaelis-Menten equation is therefore proposed to be due to erratic enzyme activity resulting in distortion of the shape of the digestion profile rather than a problem with assay sensitivity.

3.6 Investigation of the effect of the method used for Cremophor addition (direct or premixed) to the assay upon subsequent activity of pancreatin.

3.6.1 Introduction

Preliminary investigations of the activity of pancreatin towards MCT in the presence of Cremophor had revealed different results according to the method used for preparation of the reaction mixture. When preparing the substrate of MCT and Cremophor the two components have to be incorporated into the simulated bile solution. This can be achieved in two ways, by addition of MCT and Cremophor individually or by use of a combined premixed form of the two as described in the method for the standard pH-stat assay (section 2.2.3).

When using an identical concentration of Cremophor the extent of digestion was observed to relate to the method used to prepare the substrate / surfactant mixture. To investigate this phenomenon further a series of experiments were designed to quantify the change in inhibitory activity of Cremophor according to the method used to add the substrate and surfactant to the reaction mixture.

3.6.2 Method

Two methods of preparing the substrate / surfactant mixtures were investigated, the premixed method used in the standard pH-stat assay and the direct addition method. Substrate concentration was fixed at 1 g MCT to enable examination of the effect of varying the Cremophor concentration included in the reaction mixture. The quantity of Cremophor used ranged from 0.01 g in 12 stages up to 2 g, which resulted in twelve digestion profiles being obtained for each of the two methods.
For the premixed method Cremophor and MCT were combined and added to the reaction vessel as per the method described in the standard pH-stat assay (section 2.2.3). The direct addition method involved addition of the Cremophor by weight directly into a beaker followed by some simulated bile solution. A magnetic stirrer was used to incorporate the Cremophor into the simulated bile solution. The resulting solution was placed in the reaction vessel followed by separate addition of the MCT. The assay was carried out in the same manner as the standard pH-stat assay from the stage of addition of MCT to the reaction vessel.

3.6.3 Results

The digestion profiles of percent triglyceride digested against time were grouped according to the method of substrate preparation used, direct addition of Cremophor into simulated bile solution (Figure 3.15) or premixed with MCT (Figure 3.16). This allowed examination of the ability of Cremophor to suppress lipolysis of MCT (1 g) in relation to the concentration of Cremophor present in the reaction mixture and the method of preparation used.

From Figure 3.15 the inclusion of Cremophor via the direct addition method up to a concentration of 0.25 g had no effect upon triglyceride digestion when compared to the MCT control profile. However in the case of the premixed method (Figure 3.16) the profiles including Cremophor up to concentration of 0.25 g showed a decrease in the rate of digestion.

In the case of the premixed method, a Cremophor concentration of between 0.25 g and 0.5 g resulted in a dramatic decrease in extent of lipolysis with almost complete suppression reached with 1 g of Cremophor (Figure 3.16). Examination of the digestion profiles produced via the direct addition method revealed a gradual decrease in pancreatin activity with the use of Cremophor concentrations between 0.5 g and 1.9 g, with a Cremophor concentration of 2 g necessary to almost completely suppress pancreatin activity (Figure 3.15).

The same concentration of Cremophor was therefore able to exhibit a different effect upon the activity of pancreatin according to the method used for Cremophor addition to the reaction vessel. When premixed with MCT, Cremophor was capable of a greater degree of pancreatin suppression than when added directly to the simulated bile solution.

3.6.4 Discussion

The difference in the inhibitory effect of Cremophor according to the method used to prepare the assay constituents is assumed to be due to the distribution of Cremophor within the reaction mixture, and reflect the non-equilibrium style of the reaction mixture. When Cremophor is added by the direct addition method it is possible that the Cremophor molecules remain in solution or aggregate together in a different phase to the lipid substrate. However when Cremophor is added by the premixed method lipid and Cremophor have definite contact from the start of the assay and may form a phase to which lipase and / or colipase find adsorption difficult or which water has trouble penetrating.

Workers investigating the inhibition of another lipolytic enzyme[164], phospholipase C, by substrate analogs observed a long lag period in enzyme activity towards lecithin. However the lag phase did not occur if the substrate analogs were added after lecithin

hydrolysis had commenced. The initial binding of substrate to enzyme was proposed to result in an conformational change in the enzyme which increased enzyme affinity for the substrate. Inhibitory analogs present in the system before substrate hydrolysis commenced blocked the enzyme-substrate binding site, thus preventing a conformational change in the enzyme.

A similarity may be drawn between the above observation and the work performed in this investigation when using different methods of adding Cremophor to the assay. The enhanced inhibitory action of Cremophor when mixed directly with MCT could be due to the increased proximity of Cremophor and MCT at the substrate interface. Enzyme orientated at the interface would be more likely to interact with the Cremophor or Cremophor may restrict binding of lipase or colipase to the MCT. The quantity of enzyme able to bind with MCT would therefore be reduced initially resulting in depressed lipolytic activity.

In the case of Cremophor mixed directly into the simulated bile solution there is less opportunity for Cremophor to come into contact with the substrate surface or enzyme at the beginning of the reaction, as it immediately undergoes dilution in the aqueous milieu. A higher concentration of Cremophor would therefore be necessary to increase interaction of Cremophor with MCT and enzyme to produce the same degree of inhibition as given by the premixed method.

3.7 Conclusions

The quantitative investigation of pancreatin activity towards MCT indicated an apparent compliance of kinetic behaviour with the Michaelis-Menten equation (Equation 3.1). This allowed determination of the kinetic parameters K_m and V_{max} for pancreatin when acting upon MCT under conditions of the standard pH-stat assay. The values of K_m and V_{max} are apparent due to complications involved with the parameter of substrate concentration. The relationship between initial velocity of pancreatin activity towards MCT and substrate concentration may equally be reflecting a relationship between another parameter dependent on bulk substrate concentration. These parameters could include substrate availability at the interface, lipid packing density and conformation of the lipid at the interface.

For this study substrate was presented in emulsion form to increase representation of the physiological situation and provide maximum interfacial area of substrate. If the interfacial area is not present in excess to the concentration of enzyme used, initial velocity measurements and hence the values of K_m and V_{max} will be related to the increase in interfacial area with substrate concentration rather than the catalytic step. Information as to the physical state of the substrate in terms of availability to pancreatin and concentration of the substrate at the interface could increase the understanding of lipolysis kinetics further, however the values of K_m and V_{max} determined relate to the particular reaction conditions used in the standard pH-stat assay. These values were considered sufficient for use as a comparative measure of pancreatin activity for subsequent investigations involving inclusion of a surfactant with inhibitory properties within the same reaction system.

The surfactant, Cremophor, a known inhibitor of pancreatin activity towards MCT, was chosen to investigate inhibition of pancreatin activity by surfactants. The study revealed a change in the kinetic behaviour of pancreatin as a lag phase was observed when Cremophor was present in the system. The inhibition observed was examined by production of Lineweaver-Burk plots ($1/v$ against 1/[S]) in the presence of different concentrations of Cremophor, by comparison of the kinetic parameters K_m and V_{max} with those from MCT alone and through the use of the parameter of the degree of inhibition.

A linear relationship between $1/v$ against 1/[S] indicates compliance of enzyme kinetic behaviour with the Michaelis-Menten equation and is necessary to enable information regarding the mechanism of inhibition to be obtained from the Lineweaver-Burk plot. As a linear relationship between $1/v$ against 1/[S] could not be established from the experimental data it was not possible to determine if pancreatin obeyed the Michaelis-Menten equation in the presence of Cremophor, although for the plot produced in the presence of the lowest concentration of Cremophor linear behaviour appeared likely. Lack of linearity may have been a result of experimental error introduced by the lag phase at the beginning of the digestion profiles (which complicated the measurement of initial velocity) or from the use of different batches of pancreatin. Scatter in the data prevented identification of a definite mechanism for Cremophor inhibition of pancreatin. A conclusion could be drawn however that Cremophor was acting as a

reversible inhibitor due to the ability of enzyme to exhibit increased activity after the initial lag phase.

Possible mechanisms responsible for the inhibitory effect displayed by Cremophor can however be discussed from various traits in pancreatin kinetic behaviour revealed during the investigation. There are several sites at which Cremophor may act within the reaction pathway in order to produce a reduction in pancreatin activity, namely at the site of the lipid interface or at the binding site of the enzyme.

A study was made of different preparation methods for MCT and Cremophor mixtures before inclusion in the assay system. This revealed a higher inhibitory effect was given by Cremophor if premixed with MCT before addition to the aqueous medium of the reaction system. As this method increases direct contact between substrate and Cremophor, evidence is thus provided for the inhibitory action of Cremophor to be mainly related to its presence at the lipid interface rather than on the solution state of the enzyme.

At the interface Cremophor may form a layer over the MCT molecules or become incorporated into the interface. If Cremophor is presumed to form a mixed interface with MCT the resulting composite interface may have decreased susceptibility to enzymatic binding and attack than the free substrate resulting in a decreased reaction rate. The inclusion of Cremophor into the interface could also slow down pancreatin activity through an effect of substrate dilution. Alternatively the presence of Cremophor on the surface of the interface could prevent or slow down the process of enzyme adsorption by steric hindrance.

It is possible that Cremophor is interacting with colipase or lipase at the lipid or other binding sites on the enzyme. Lipase may have a high affinity to bind with Cremophor as a substrate analog whereupon binding is followed by slow hydrolysis of Cremophor relieving the inhibition with time. This theory would account for the existence of the lag phase which increases in duration with Cremophor concentration, as initially more enzyme would be bound in the complex form unavailable to act upon the natural substrate.

An increase in enzyme activity with time allows proposals of mechanisms by which inhibition due to Cremophor may be relieved. As lipolysis slowly proceeds the products released such as fatty acids and monoglycerides may act to remove

Cremophor from the system by forming a complex. Alternatively they may enhance enzyme binding by increasing colipase partitioning into the interface. Cremophor itself may gradually change phases leaving the substrate more available for hydrolysis. If the phase transition is abrupt kinetic behaviour of the enzyme will change dramatically if the physical state of the substrate is the factor limiting lipolysis.

In summary this study has revealed that Cremophor is acting as a reversible inhibitor with the suppression of pancreatin activity increased when a method involving close contact between Cremophor and substrate is used for assay preparation. This suggests the inhibitory mechanism of Cremophor is related mainly to the lipid interface, with the lag phase representing a delay in the initial adsorption of enzyme to substrate due to Cremophor altering the physical state of the substrate.

Cremophor does not completely prevent all enzyme / lipid binding as a limited amount of lipid hydrolysis is seen to occur during the lag phase. The released lipolytic products may accumulate at the triglyceride interface to enhance colipase partitioning into the interface, displacing some of the Cremophor molecules in the process. This mechanism known as product activation has the overall effect of a sudden increase in hydrolytic rate leading to the biphasic kinetics seen.

As Cremophor could be decreasing pancreatin activity through a combination of effects this would explain the lack of a linear relationship between initial velocity and substrate concentration. The kinetic data obtained from this study is too complex for determination of the inhibitory mechanism of Cremophor to be possible via the methods used. Scatter in the results, although in some part due to experimental error, may also be related to the erratic kinetic behaviour of lipase resulting from rapid phase distribution changes of substrate, surfactant and enzyme within the system. The composite nature of Cremophor may also increase the discontinuity at the interface and thus differences in kinetic activity.

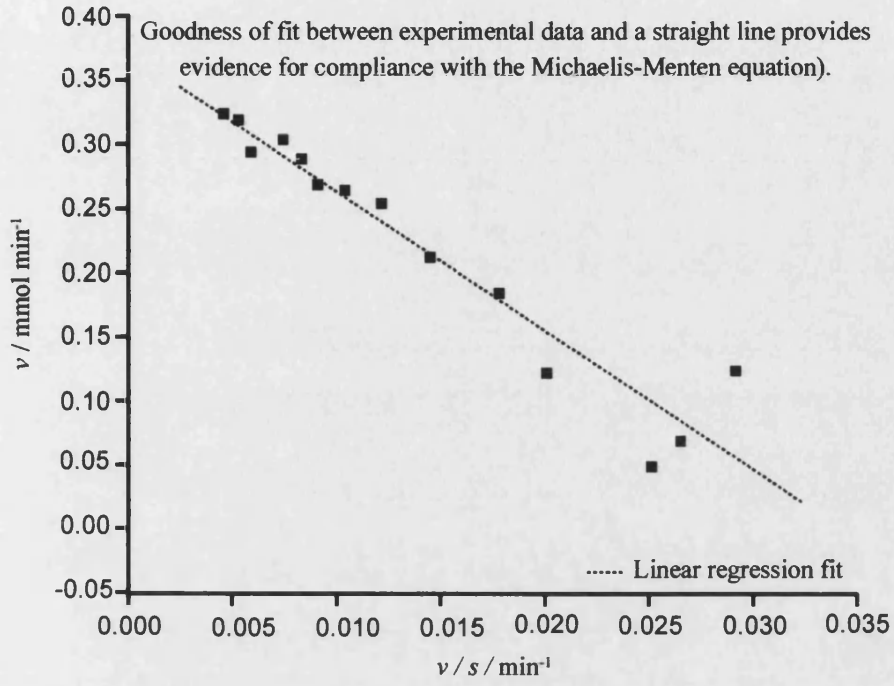
Relevance *in vivo.*

The standard pH-stat assay has been used throughout this investigation as an *in vitro* model of lipolysis. A point for consideration is that kinetic behaviour of lipase can vary tremendously with small changes in reaction conditions[136]. This investigation has demonstrated a rapid change in kinetic behaviour by the sudden end of the lag phase

followed by rapid activity on some digestion profiles. The reason for the end of the lag phase is unknown therefore lipase may act towards the same substrate / surfactant combination under dynamic *in vivo* conditions quite differently. However it is reasonable to conclude that a substance acting as an inhibitor *in vitro* is likely to exhibit an inhibitory effect, if not to the same degree, *in vivo*.

The concept of accumulation of lipolytic products at the interface increasing colipase partitioning and hence lipolytic activity can also be related to the physiological situation[139]. When dietary triglycerides reach the duodenum they have already been partially hydrolyzed in the stomach by human gastric lipase. The lipolytic products released are already available to enhance binding of colipase and thus facilitate lipolysis in the small intestine. This may lead to any surfactant-mediated inhibition being overcome *in vivo* before the formulation has reached the duodenum. However for a highly inhibitory surfactant the lag phase *in vivo* may be greater than the GI transit time of the formulation thus preventing drug release from a lipid-based formulation via digestion of the triglyceride.

Figure 3.1 A linear arrangement of the Michaelis-Menten equation (Eadie-Hofstee plot *v* versus *v/s*).



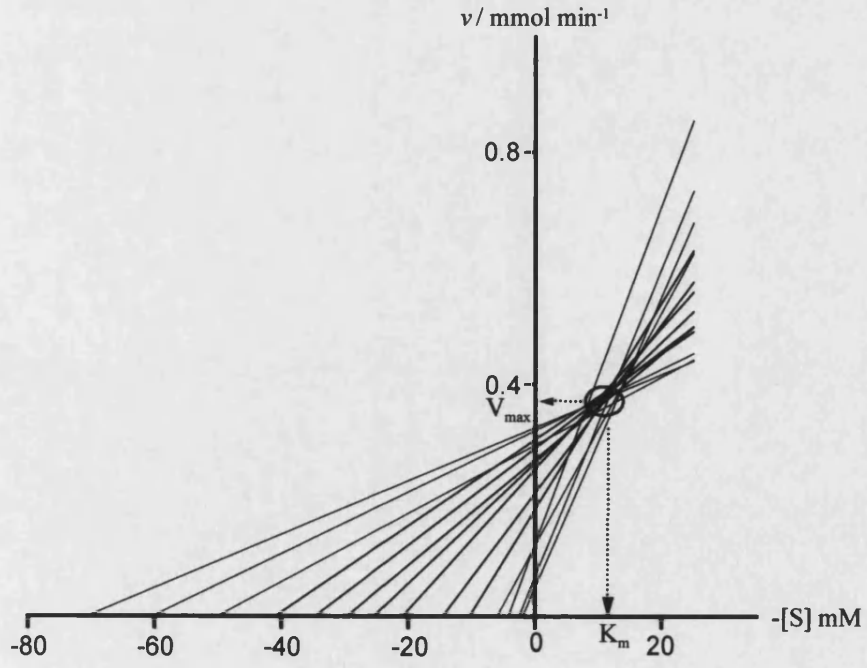


Figure 3.2 Direct linear plot for the digestion of MCT by pancreatin.

This plot illustrates the direct linear method, V_{max} and K_m are determined from the best fit median values of the intercepts.

Figure 3.3 Computerized least squares fit of a *v* against *s* hyperbola to experimental data from the hydrolysis of MCT by pancreatin.

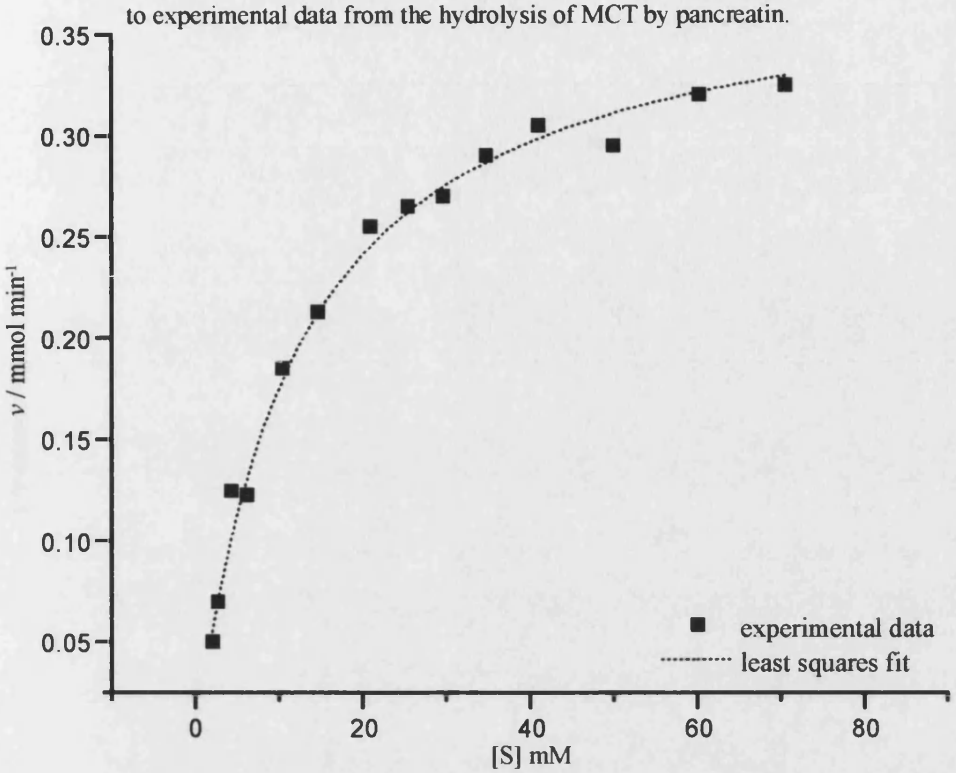


Figure 3.4 Illustration of differences in the nature of the digestion profiles produced by pancreatin when hydrolysing MCT in the presence of Cremophor.

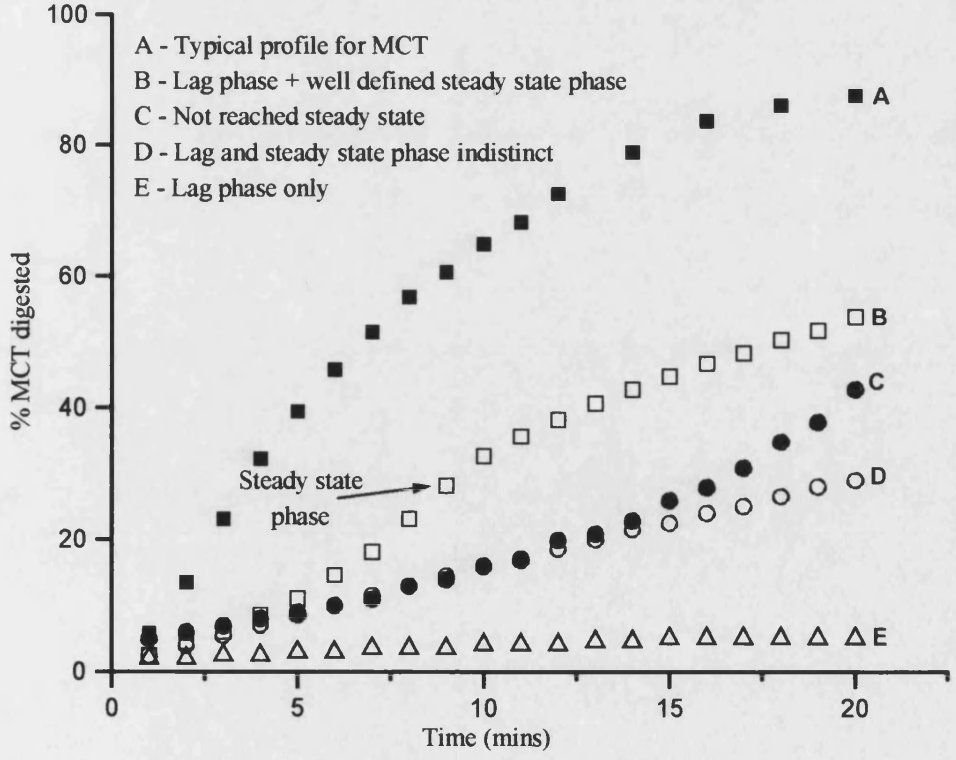


Figure 3.5a The $1/v$ versus 1/[S] plot for the hydrolysis of MCT by pancreatin in the presence of Cremophor (10 mg).

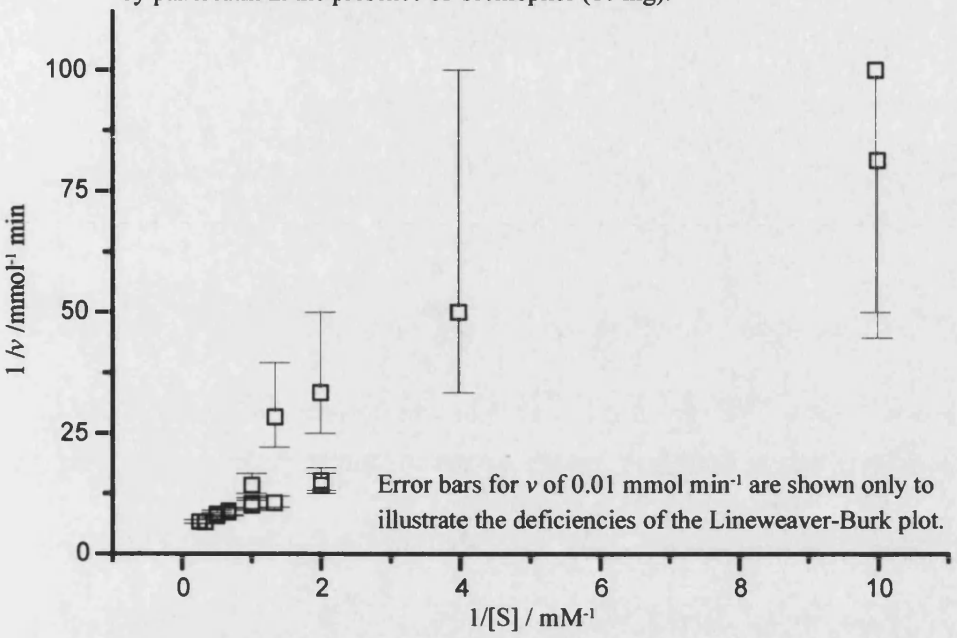


Figure 3.5b The $1/v$ versus 1/[S] plot for the hydrolysis of MCT by pancreatin in the presence of Cremophor (50 mg).

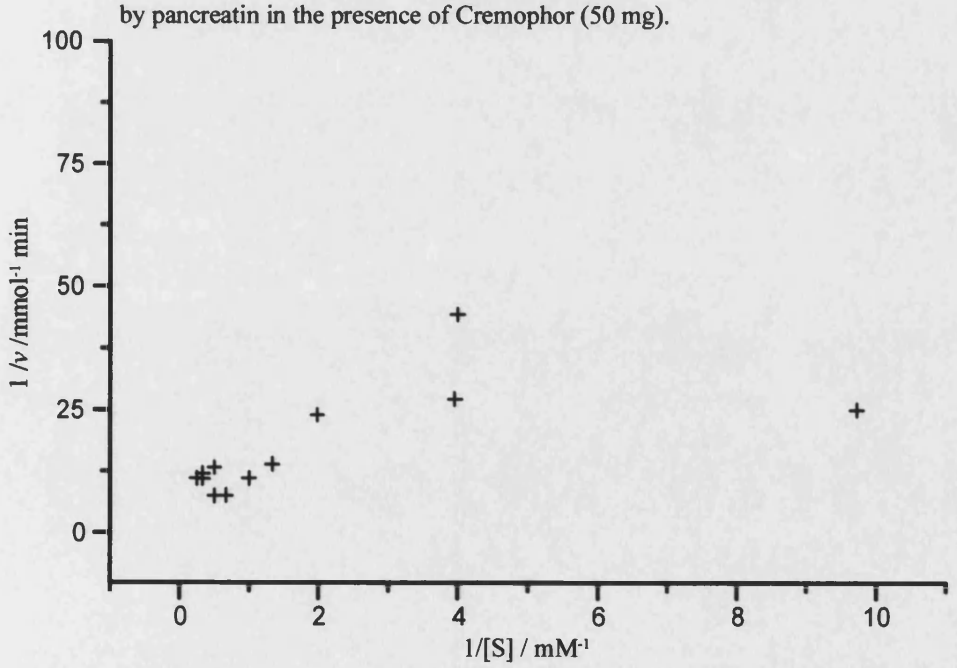


Figure 3.5c The 1/*v* versus 1/[S] plot for the hydrolysis of MCT by pancreatin in the presence of Cremophor (100 mg).

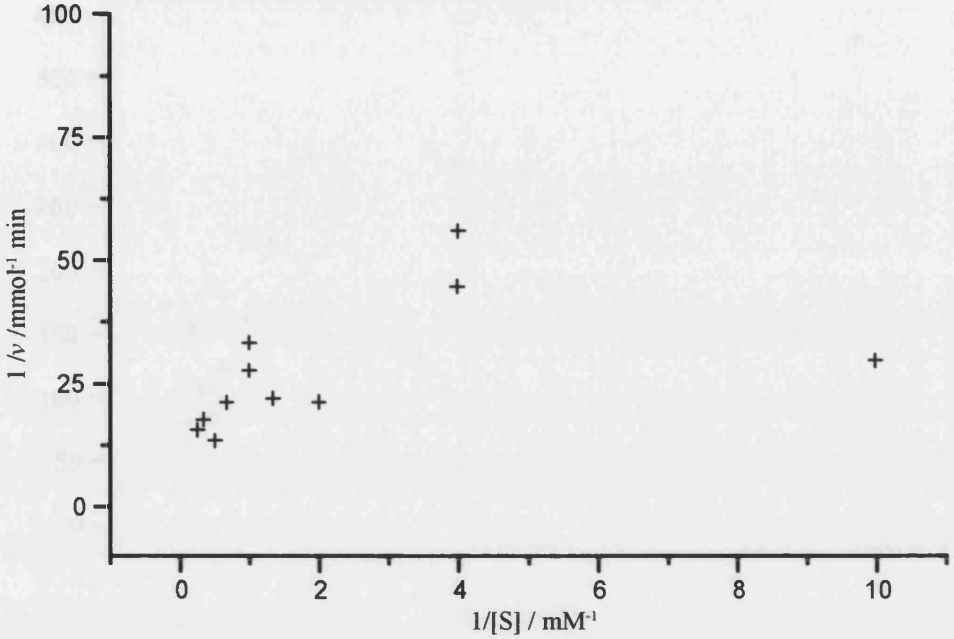


Figure 3.5d The 1/*v* versus 1/[S] plot for the hydrolysis of MCT by pancreatin in the presence of Cremophor (250 mg).

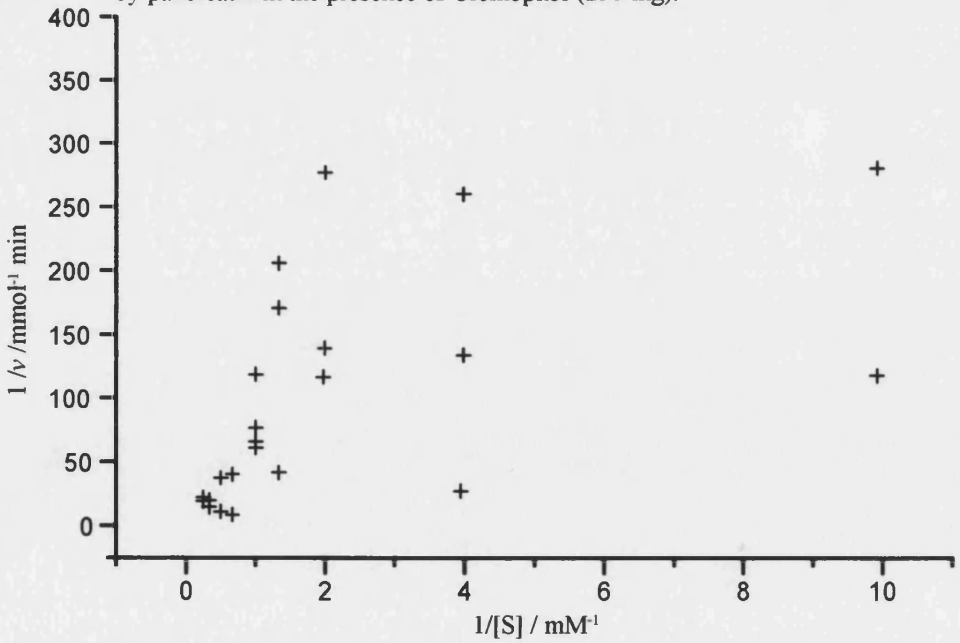


Figure 3.5e The $1/v$ versus 1/[S] plot for the hydrolysis of MCT by pancreatin in the presence of Cremophor (500 mg).

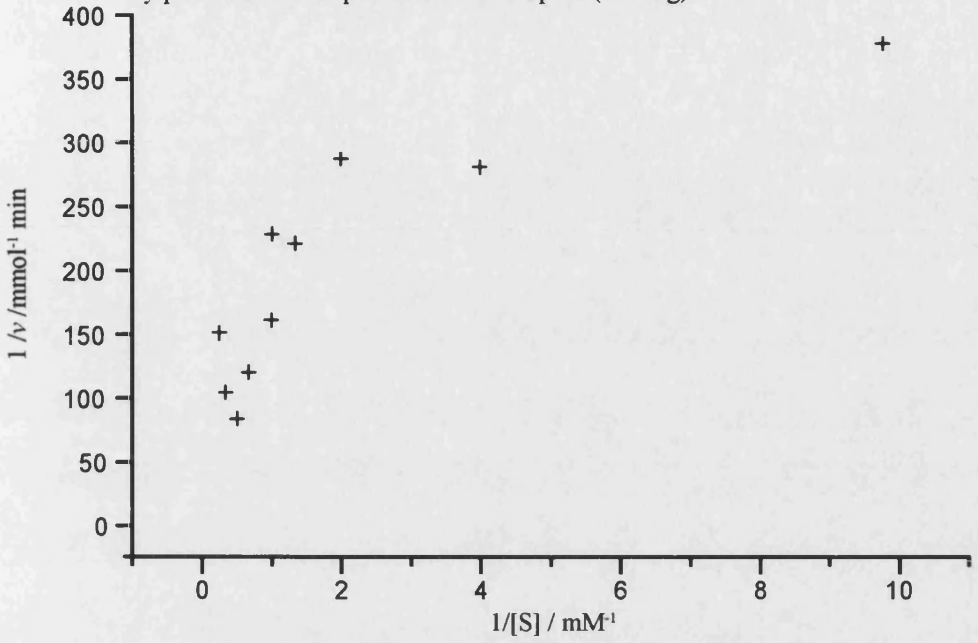


Figure 3.6 The combined 1/*v* versus 1/[S] plot for the hydrolysis of MCT by pancreatin in the presence of different fixed concentrations of Cremophor.

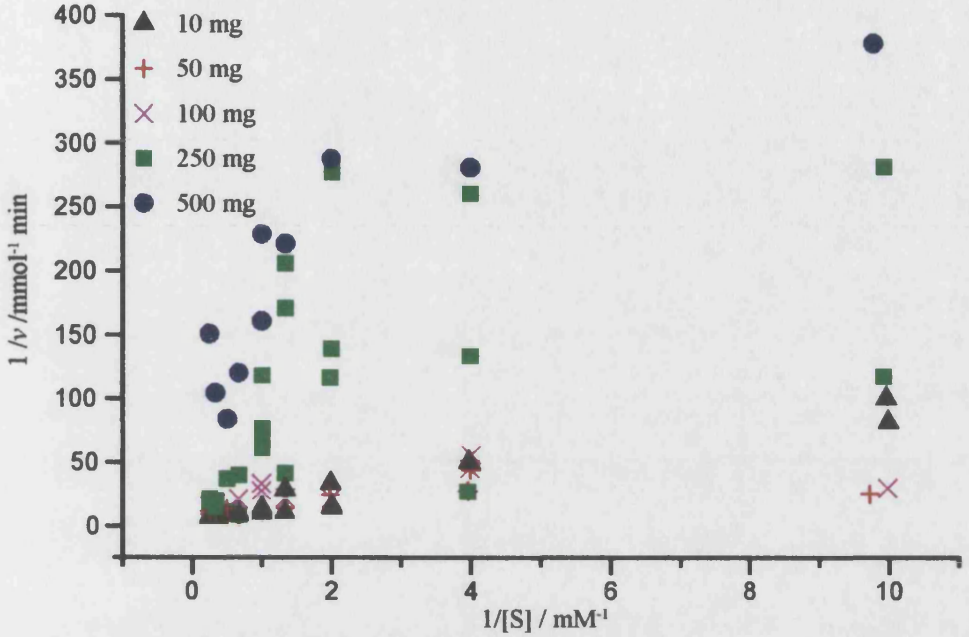


Figure 3.7 Computerized least squares fit of a *v* against S hyperbola to experimental data from the hydrolysis of MCT by pancreatin in the presence of Cremophor (10 mg).

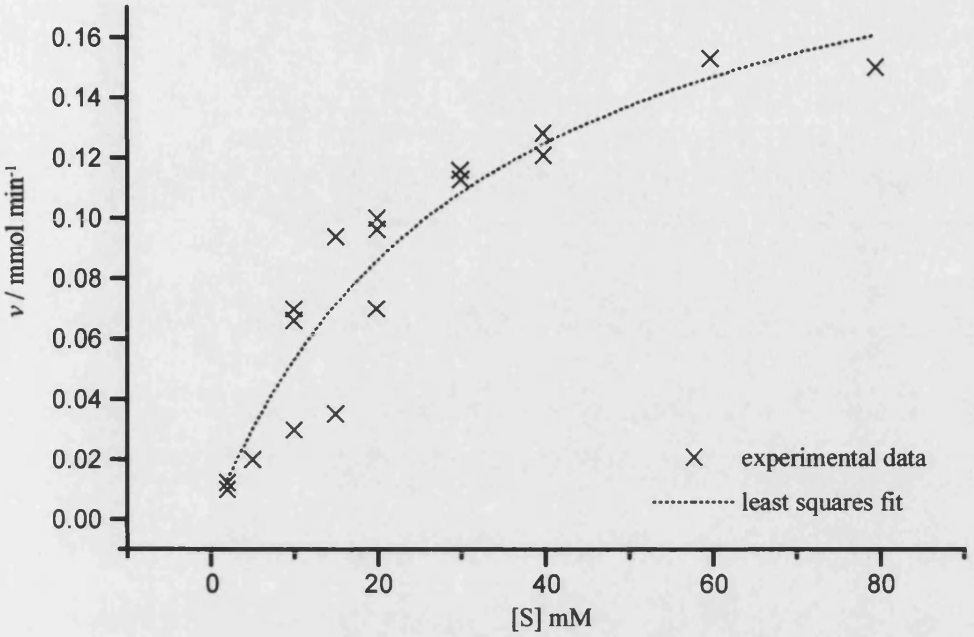


Figure 3.8 The effect of an increase in substrate concentration on pancreatin activity in the presence of a fixed concentration of Cremophor (10 mg).

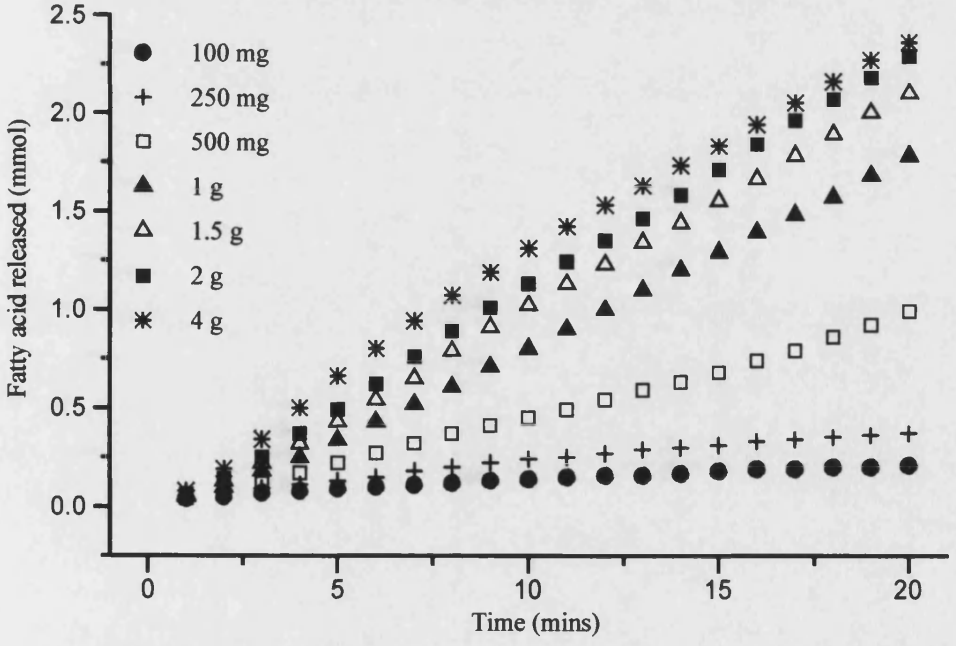


Figure 3.9a Comparison of uninhibited initial velocity ($V_{\%}$) as a function of substrate concentration in the presence of different fixed concentrations of Cremophor.

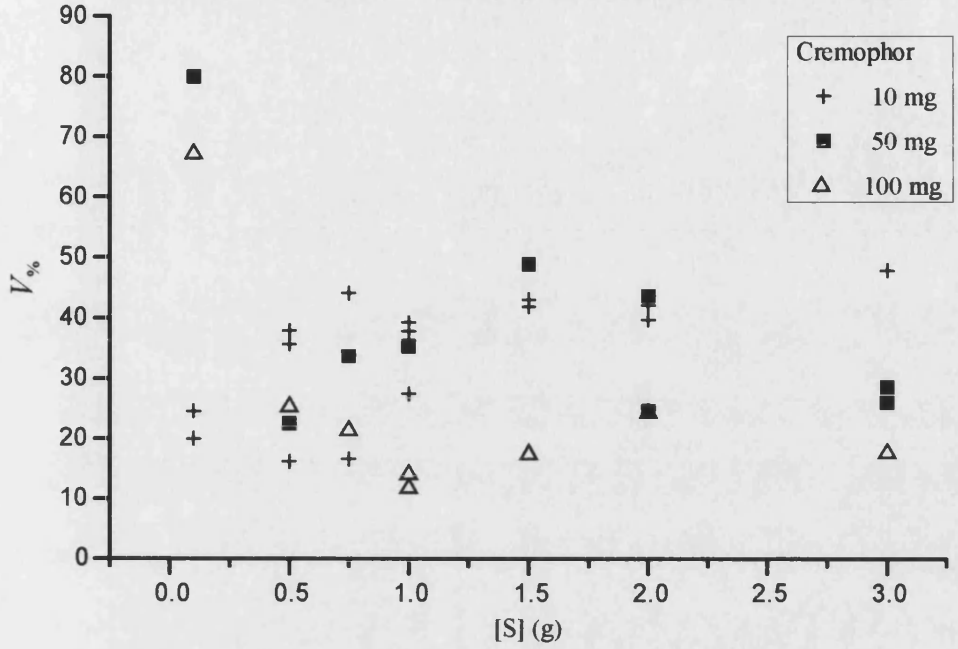


Figure 3.9b Comparison of uninhibited initial velocity ($V_{\%}$) as a function of substrate concentration in the presence of different fixed concentrations of Cremophor.

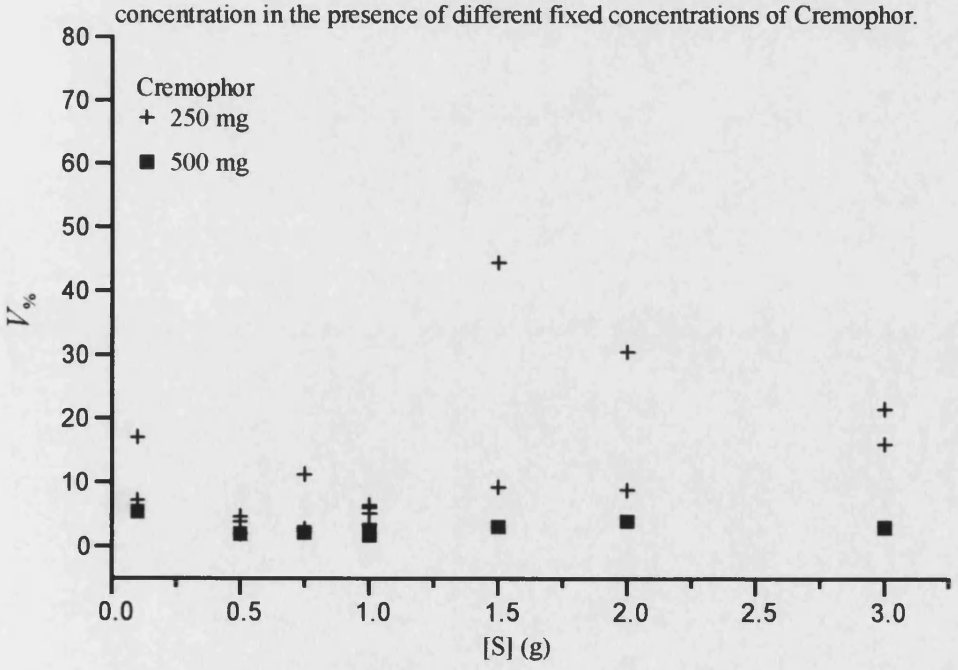


Figure 3.10 The effect of an increase in Cremophor concentration on pancreatin activity towards a fixed concentration of substrate (MCT 1 g).

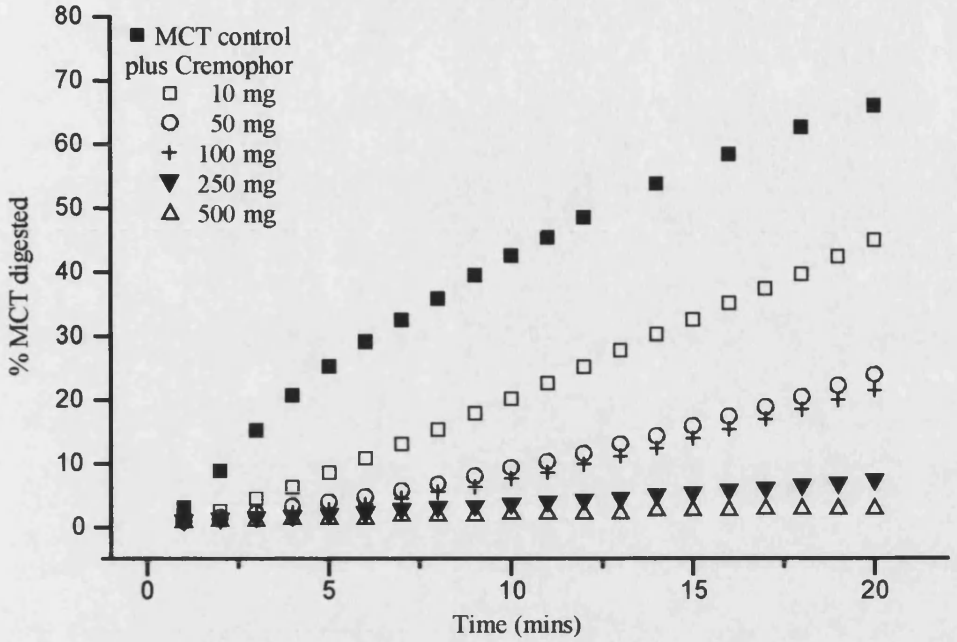


Figure 3.11 Time taken to digest 2.5 % of substrate (MCT 1 g) in the presence of different concentrations of Cremophor. (Calculated from digestion profiles in Figure 3.10).

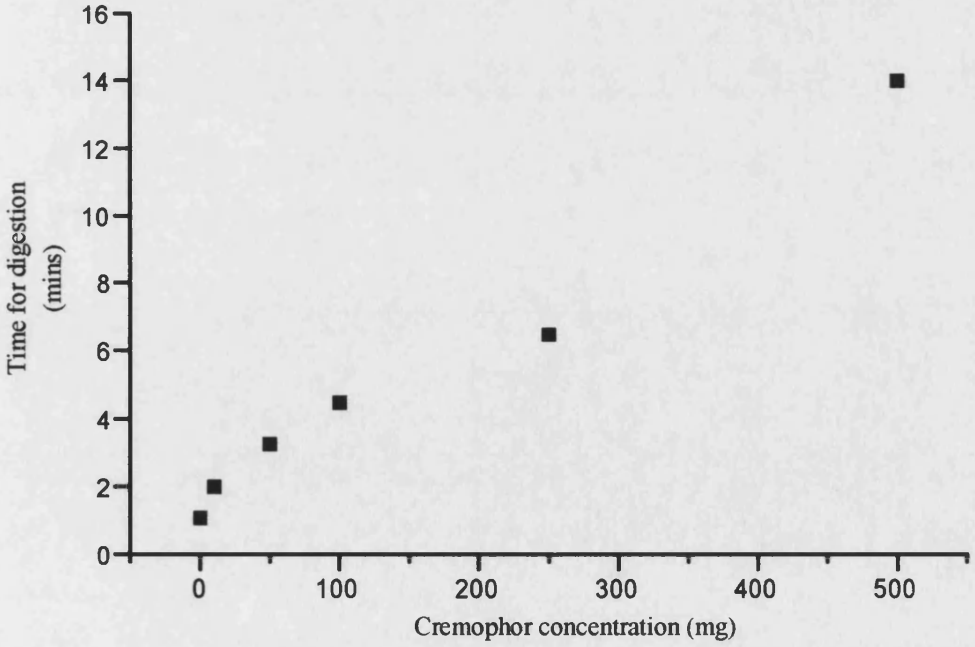
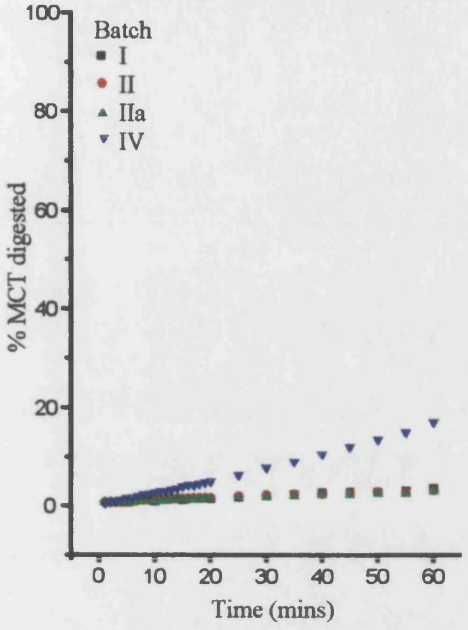
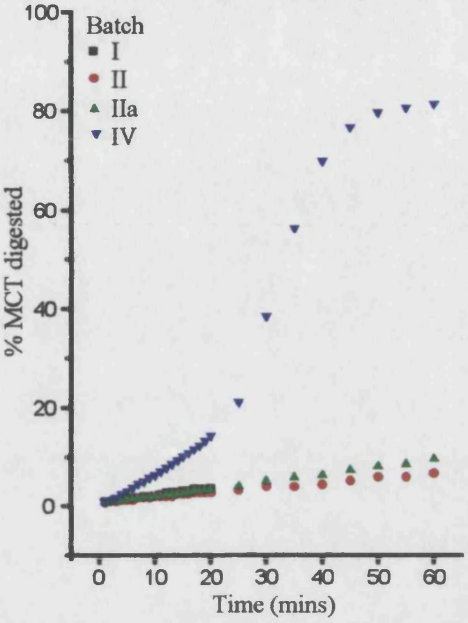


Figure 3.12(a-d) Comparison of enzyme activity shown by different batches of pancreatin towards a substrate of :

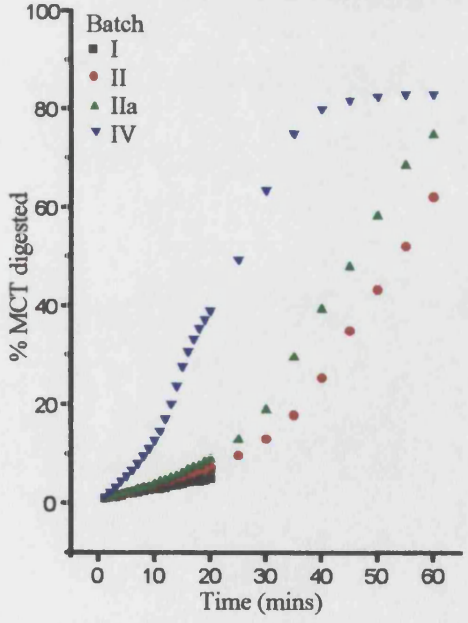
a - MCT (1 g) and Cremophor (1 g).



b - MCT (1 g) and Cremophor (500 mg).



c - MCT (1 g) and Cremophor (250 mg).



d - MCT (1 g) and Cremophor (100 mg).

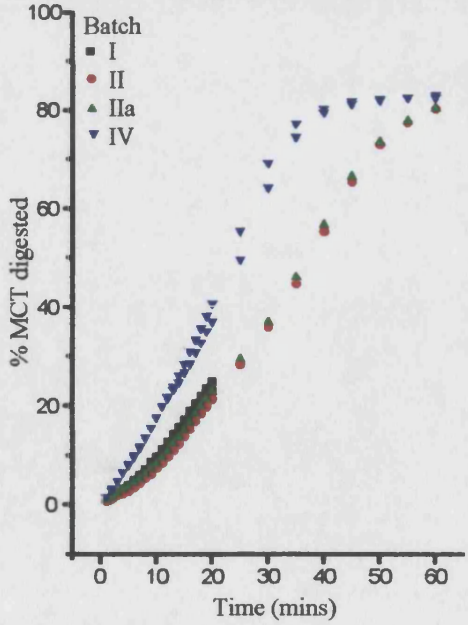
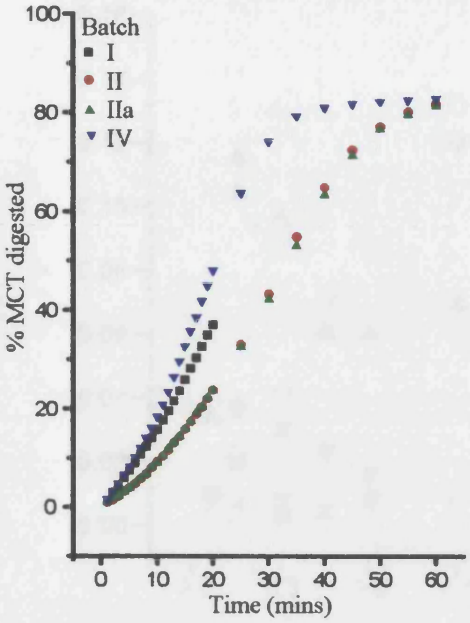
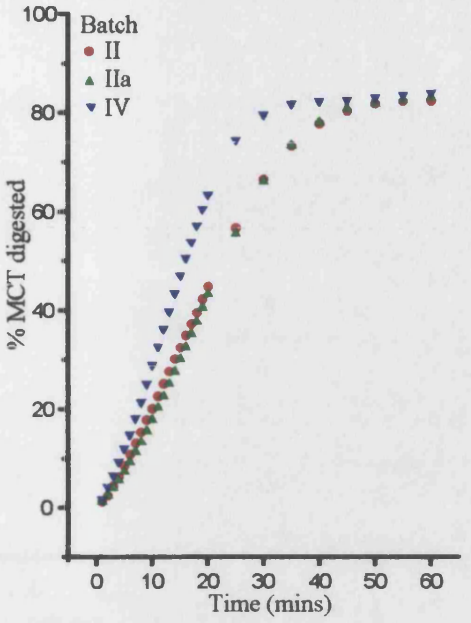


Figure 3.12(e-g) Comparison of enzyme activity shown by different batches of pancreatin towards a substrate of :

e - MCT (1 g) and Cremophor (50 mg).



f - MCT (1 g) and Cremophor (10 mg).



g - MCT (500 mg) and Cremophor (10 mg).

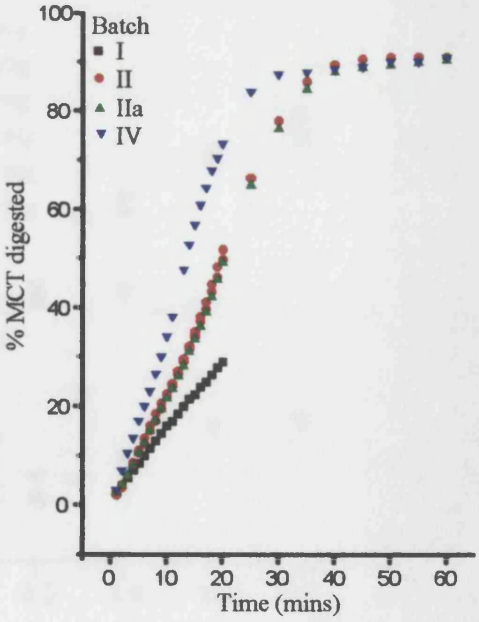


Figure 3.13a The combined *v* versus [S] plot for the hydrolysis of MCT by pancreatin (Batch I) in the presence of different fixed concentrations of Cremophor.

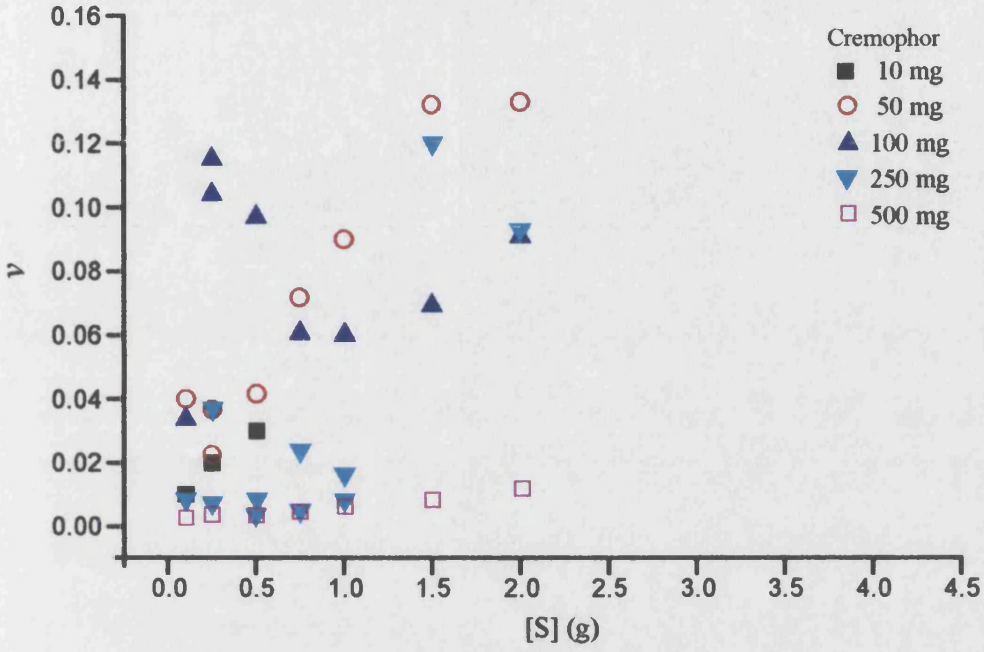


Figure 3.13b The combined *v* versus [S] plot for the hydrolysis of MCT by pancreatin (Batch II) in the presence of different fixed concentrations of Cremophor.

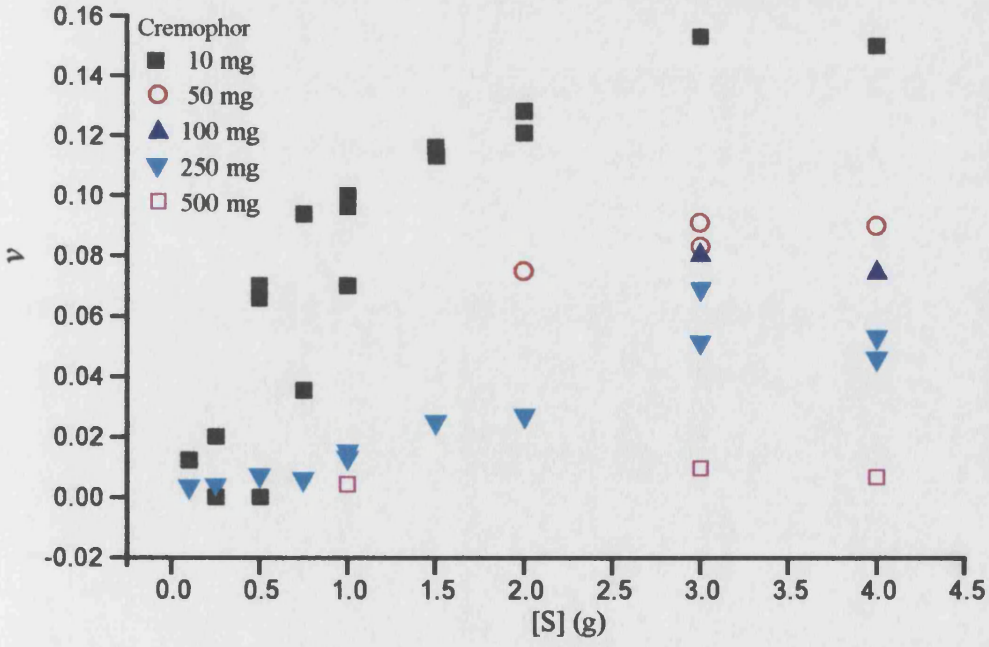


Figure 3.14 Computerized least squares fit of a v against S hyperbola to experimental data from the hydrolysis of MCT by pancreatin in the presence of Cremophor (10 mg) using 0.1 M NaOH as titrant.

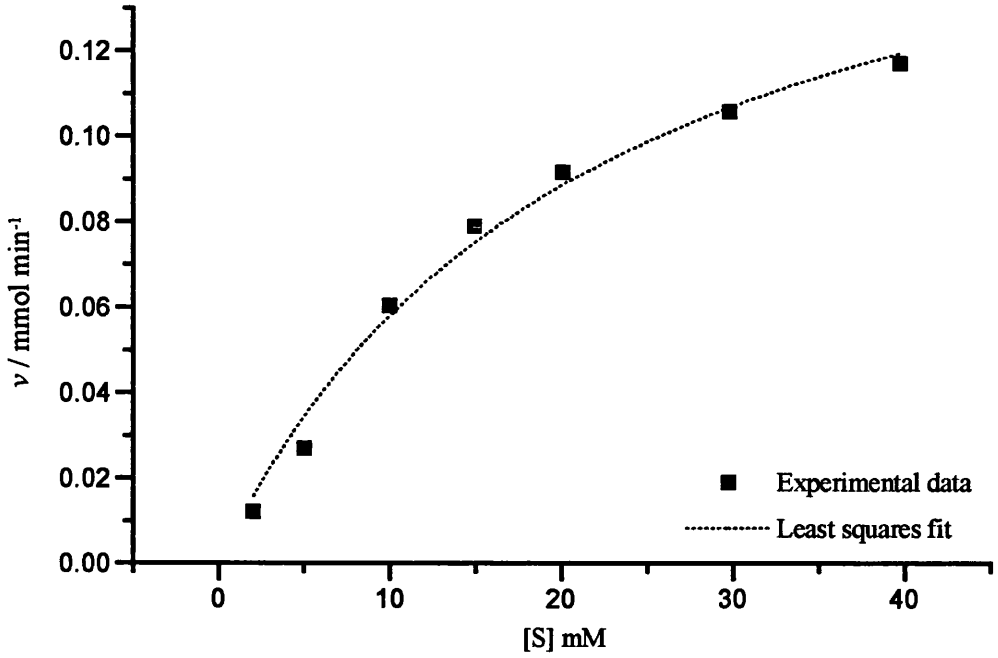


Figure 3.15 Digestion profiles of MCT (1 g) in the presence of Cremophor when added to the assay system via the **direct addition method**.

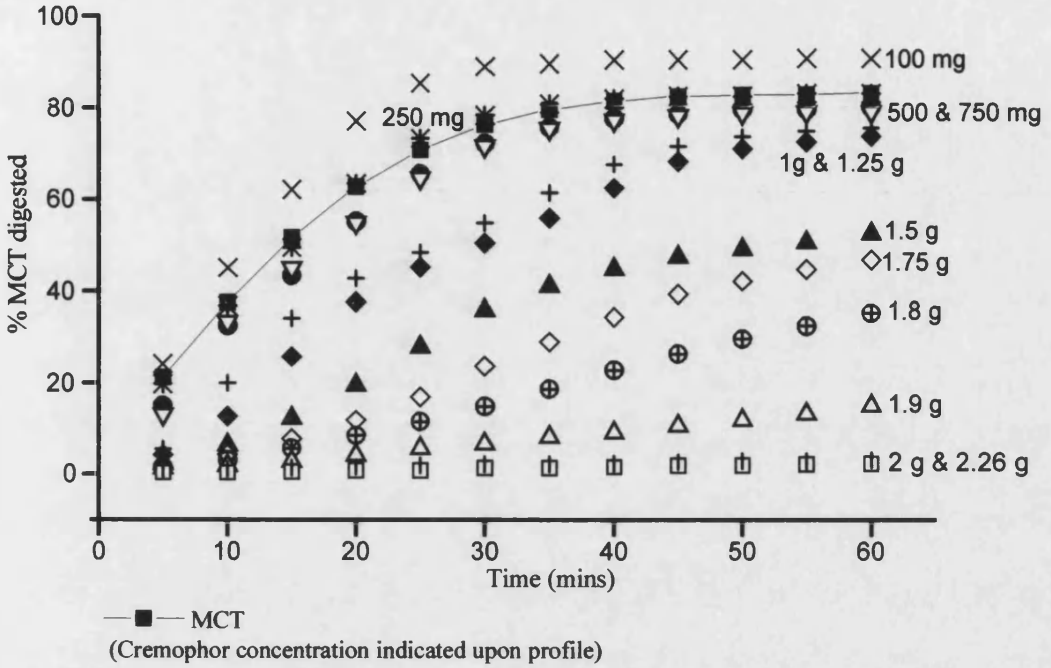
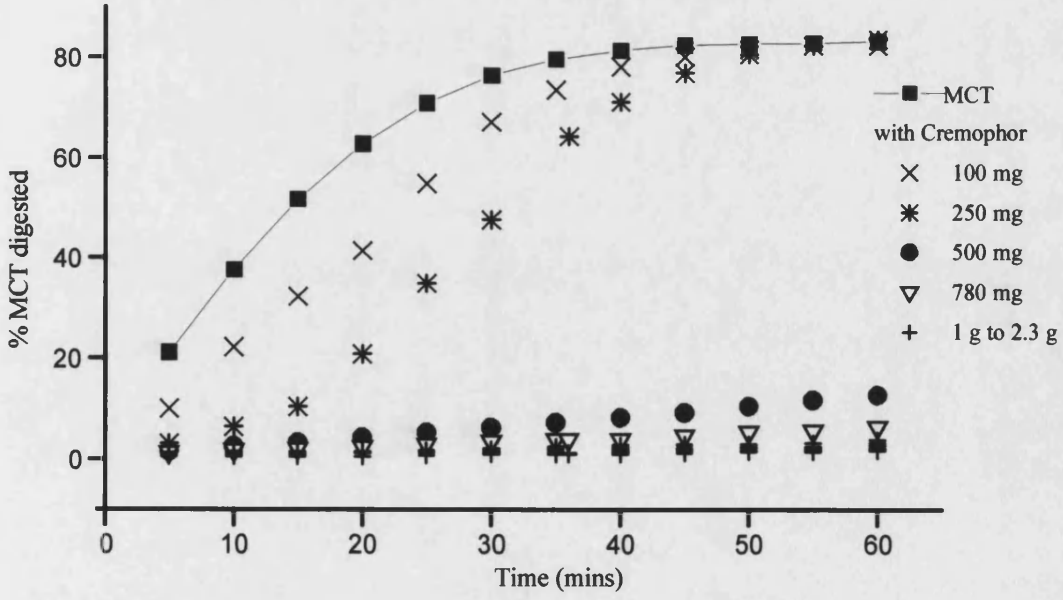


Figure 3.16 Digestion profiles of MCT (1 g) in the presence of Cremophor when added to the assay system via the **premixed method**.



Chapter 4 - Inhibition of pancreatin activity towards MCT by non-ionic surfactants: A structure-activity study.

4.1 Introduction

The non-ionic surfactant, Cremophor was shown to depress the rate of digestion of MCT by pancreatin under conditions of the standard pH-stat assay in Chapter 3. Information as to which non-ionic surfactant types are likely to inhibit pancreatin activity would be useful so their use in lipid-based formulations could be avoided. This is desirable due to the suspected role of lipid digestion in enhancement of the bioavailability of hydrophobic drugs from lipid-based formulations.
Pancreatin acts at the interface of a lipid / water system therefore accumulation of surfactant at the interface could alter enzyme activity. The features of a surfactant which were expected to affect the activity of pancreatin were the chemical structure and the balance between the hydrophilic and lipophilic properties of a surfactant. If a relationship was found to exist between a particular surfactant property and potency of inhibition towards pancreatin this may provide further information regarding the mechanism by which non-ionic surfactants inhibit pancreatin activity.

4.1.1 Characteristics of surfactants.

Surface active agents, commonly known as surfactants, are molecules comprised of two distinct regions[165]. One of the regions is hydrophobic and consists of hydrocarbon chain or ring systems with saturated or unsaturated bonds. The other region is hydrophilic, and may contain an ionized group which confers an electrical charge on the surfactant molecule.
Surfactants where the hydrophilic moiety consists of polyoxyethylene chains or hydroxyl groups have no residual electrical charge on the molecule hence they are known as non-ionic. Non-ionic surfactants are preferred for pharmaceutical purposes due to their reduced toxicity compared to other surfactant groups and lack of sensitivity to other components likely to be present in a lipid-based formulation[165]. All

investigations and discussion of surfactant behaviour in this thesis relates to the non-ionic group.

As a surfactant has two moieties, one of which has affinity for the solvent whilst the other does not, the conferred dual nature of the molecule is described as amphipathy[166]. It is the amphipathic nature of surfactants which is responsible for their tendency to accumulate at the surface boundary between two phases. This ultimately can lead to surface activity at liquid / liquid or liquid / air interfaces, micellization and solubilization.

4.1.2 The hydrophile-lipophile balance (HLB) of a surfactant.

As the behaviour of surfactants is highly dependent on the respective influence from their hydrophilic and lipophilic moieties, various methods have been devised to quantify the balance between the two. The HLB number method, devised in 1949 by Griffin[167], has been widely adopted as an empirical method of representing the balance between the hydrophilic and lipophilic content of a surfactant molecule. Griffin later adapted the concept[168] into a series of equations which enable calculation of surfactant HLB numbers using analytical or composition data.

Equation 4.1[168] is commonly used to determine the HLB number for polyhydric alcohol fatty acid esters for which saponification numbers can be obtained analytically.

$$\text{HLB} = 20\left(1 - \frac{S}{A}\right) \qquad \text{Equation 4.1}$$

where S = Saponification number of the ester.

A = Acid number of the fatty acid which is defined as the number of mg of KOH required to neutralize 1 g of fatty acid.

If saponification data is unavailable or difficult to obtain, Equation 4.2[168] allows calculation of the surfactant HLB value from compositional data if the hydrophilic surfactant moiety consists of polyhydric alcohol and oxyethylene groups.

$$\text{HLB} = \frac{E + P}{5} \qquad \text{Equation 4.2}$$

where E = The percentage by weight of the surfactant molecule made up by oxyethylene chains.

P = The percentage by weight of the surfactant molecule made up by polyhydric alcohol groups. This term is disregarded if the hydrophilic portion of the surfactant molecule consists solely of oxyethylene.

Another method developed by Shinoda et al[169] involves determination of the phase inversion temperature (PIT). The PIT is defined as the temperature at which the hydrophilic and lipophilic properties of the surfactant balance at the interface and maximum solubilization of oil occurs[170]. Whereas the HLB number remains constant for a particular surfactant as it is assigned via surfactant structure, the PIT is a characteristic property of the emulsion system. The PIT will therefore alter to reflect changes in the behaviour of a particular surfactant due to influences from the emulsion system such as ionic strength, temperature, structural modifications of the surfactant molecule, other additives in the system, and nature of the oil phase[171].
Although the HLB number provides no information regarding influence from the environment upon behaviour of the surfactant, it is widely used to select surfactants suitable for a particular purpose such as emulsification, solubilization or detergency. For non-ionic surfactants the HLB number ranges from 0 to 20 on an arbitrary scale[165]. The lower numbers on the scale (0 to 5) represent surfactants with high lipophilicity which can be used as water in oil emulsifiers and antifoaming agents. HLB numbers in the range of 7 to 16 approximately are oil in water emulsifiers with HLB values from 13 to 17 indicating surfactants with detergent properties. Surfactants with HLB values from 17 upwards are very hydrophilic and act as solubilising agents.

4.1.3 Influence of the HLB value of a surfactant on emulsion systems.

The ability of a surfactant to emulsify a system of water and oil is related to the interfacial film of surfactant formed at the phase boundary. To achieve a minimum

free energy state the hydrophobic portion of the surfactant becomes orientated in the oil phase[165]. The accumulation of surfactant molecules at the interface chemically changes the nature of the interface, in addition to replacing some of the water molecules previously present. The resulting mixed interface has reduced intermolecular attraction and therefore a lower surface tension which enhances emulsion formation[165].

The type of emulsion formed, emulsion stability and orientation of the surfactant molecule in the interface is dependent on the HLB of a surfactant. As the polyoxyethylene chain is lengthened through a homologous surfactant series, hydrophilic character and the HLB number of the surfactant increases. At high HLB values the hydrophilic moiety of the surfactant dominates surfactant character with strong hydration forces between water and the polyoxyethylene chain[171]. This highly hydrated interfacial film results in a convex curvature of the interface towards water giving the surfactant properties of an oil in water emulsifier. Surfactants with a short polyoxyethylene chain exhibit low hydration effects, the interface has a concave curvature towards water and the surfactant acts as a water in oil emulsifier. At intermediate HLB values between the two states the degree of hydration will result in an interface with little curvature and at this point the HLB is said to be balanced. Surfactants with different HLB values will have varying effects on surface tension and thus ease of emulsification. If the hydrocarbon portion is extended through a homologous surfactant series, surface tension will be reduced promoting emulsification whereas if the hydrophilic region is enlarged the opposite effect is seen[165].

The HLB value of a surfactant also provides information on the likely phase behaviour of any surfactant not adsorbed at the interface. A surfactant where the lipophilic moiety is dominant (as indicated by a low HLB) will tend to dissolve in the non-polar phase of an emulsion whereas a predominantly hydrophilic surfactant would partition into the water phase. If the surfactant exhibits low solubility in both oil and water this will tend to result in formation of a separate surfactant phase in the system[171].

In terms of emulsions the properties of the interfacial film will determine the ease of emulsion formation whereas the stability of the emulsion is dependent upon interfacial tension[172] although other factors such as steric stabilization are also involved. As the

degree of surfactant ethoxylation will affect properties of the interface, an emulsion produced from an identical oil and water mixture will have different characteristics depending on HLB value of the surfactant used.

4.1.4 Outline of this investigation.

This investigation covers several aspects of the inhibition of pancreatin activity towards MCT by non-ionic surfactants. Non-ionic surfactants are ideal for investigation of the effect of surfactant polarity (expressed by the HLB value) on pancreatin activity, as HLB can be increased through a homologous series of surfactants solely by lengthening the polyoxyethylene chain. Pancreatin activity was therefore able to be assessed in the presence of surfactants covering a wide range of HLB values without alteration in the basic chemical structure of the surfactant used. A series of nonylphenol ethoxylated surfactants (NPEs) were initially employed to establish if a relationship existed between the HLB value of a surfactant and potency of inhibition shown towards pancreatin (sections 4.2 to 4.5). The toxicity profile of NPEs means they are unsuitable for pharmaceutical purposes, as their small size allows ease of penetration and disruption of lipid membranes. However they were considered ideal for the above investigation due to the large number of commercial materials available, covering a wide range of HLB values.

The study was then extended to involve homologous series of non-ionic surfactants generally considered suitable for pharmaceutical applications (sections 4.6 to 4.12). This was to determine whether the degree of ethoxylation of a surfactant was the sole influence regarding potency of inhibition or if the chemical structure of the surfactant hydrophobe was also relevant. The number of surfactants available in each series was limited which placed a restriction on the HLB values investigated. However sufficient surfactants were obtained to enable comparison of the extent of inhibition seen with results from the NPEs.

Section 4.13 describes an attempt to characterize the degree of ethoxylation for a range of surfactants included in the inhibition study, using cloud point and ^{1}H NMR analysis. This was to examine the accuracy of the extent of surfactant ethoxylation quoted by the manufacturer.

4.1.5 Materials

As for Chapters 2 and 3 with the addition of nonylphenol (Aldrich 29,085-8) and the industrial non-ionic surfactants used in this study which have been grouped into the classes described below:

- Alcohol ethoxylates (nonylphenol ethoxylates and linear alcohol ethoxylates)
- Castor oil ethoxylates and hydrogenated castor oil ethoxylates
- Sorbitan esters
- Ethoxylated esters (polyethoxylated sorbitan esters and polyglycolyzed glycerides)

The non-ionic surfactants are listed in their respective series together with the manufacturers specification for each product. The ethoxylation number represents the number of moles of ethylene oxide reacted with one mole of the respective surfactant hydrophobe; it is usually equivalent to the average number of ethoxy groups present in the polyoxyethylene chain or the average number of ethoxy groups distributed across various ethoxylation sites on the molecule. The HLB value quoted for each surfactant has been obtained from the manufacturers data sheet, or if not available from this source was calculated from the ethoxylation number via Equation 4.2.

Nonylphenol ethoxylated surfactants

Manufacturer Series	Ethoxylation number	HLB number
Arkopal		
N-040	4	8.9
N-060	6	10.9
N-080	8	12.3
N-100	10	13.3
N-110	11	13.8
N-150	15	15.0
N-230	23	16.4
N-300	30	17.1

Manufacturer Series	Ethoxylation number	HLB number
Dowfax		
9N2	2	5.7
9N4	4	8.9
9N5	5	10.0
9N6	6	10.9
9N7	7	11.7
9N7.5	7.5	12.0
9N10	10	13.3
9N12	12	14.1
Ethylan		
44	4	8.9
77	6.5	11.0
Lutensol		
AP14	14	14.7
AP20	20	16.0
Synperonic		
NP4	4	8.9
NP5	5	10.5
NP6	6	10.9
NP8	8	12.3
NP9	9	12.8
NP10	10	13.3
NP12	12	13.9
NP13	13	14.4

Linear alcohol ethoxylated surfactants

Series	Alkyl chain	Ethoxylation number	HLB number
Brij			
30	lauryl ($C_{12:0}$)	4	9.7
35	lauryl ($C_{12:0}$)	23	16.9
52	cetyl ($C_{16:0}$)	2	5.3

Series	Alkyl chain	Ethoxylation number	HLB number
56	cetyl ($C_{16:0}$)	10	12.9
58	cetyl ($C_{16:0}$)	20	15.7
72	stearyl ($C_{18:0}$)	2	4.9
76	stearyl ($C_{18:0}$)	10	12.4
78	stearyl ($C_{18:0}$)	20	15.3
92	oleyl ($C_{18:1}$)	2	4.9
96	oleyl ($C_{18:1}$)	10	12.4
98	oleyl ($C_{18:1}$)	20	15.3
Lutensol			
AO3	synthetic $C_{13:0}$ - $C_{15:0}$	3	8.0
AO8	synthetic $C_{13:0}$ - $C_{15:0}$	8	12.5
AO10	synthetic $C_{13:0}$ - $C_{15:0}$	10	13.5
AT11	fatty alcohol $C_{16:0}$ - $C_{18:0}$	11	13.0
AO12	synthetic $C_{13:0}$ - $C_{15:0}$	12	14.3

Castor oil ethoxylated surfactants

Series	Ethoxylation number	HLB number
Etocas		
5	5	3.9
10	10	6.3
35	35	12.7
40	40	13.0
60	60	14.7
100	100	16.5

Hydrogenated castor oil ethoxylated surfactants

Series	Ethoxylation number	HLB number
Croduret		
7	7	5.0
40	40	13.0
50	50	14.1
60	60	14.7

Sorbitan esters

Series			HLB number
Crill			
1	monolaurate	($C_{12:0}$)	8.6
2	monopalmitate	($C_{16:0}$)	6.7
3	monostearate	($C_{18:0}$)	4.7
4	monooleate	($C_{18:1}$)	4.3
41	tristearate	($C_{18:0}$)	2.1
43	sesquioleate	($C_{18:1}$)	3.7
45	trioleate	($C_{18:1}$)	1.8
Span			
65	tristearate	($C_{18:0}$)	2.1

Polyethoxylated sorbitan esters

Series			Ethoxylation number	HLB number
Crillet				
1	monolaurate	($C_{12:0}$)	20	16.7
2	monopalmitate	($C_{16:0}$)	20	15.6
3	monostearate	($C_{18:0}$)	20	14.9
4	monooleate	($C_{18:1}$)	20	15.0
11	monolaurate	($C_{12:0}$)	4	13.3
35	tristearate	($C_{18:0}$)	20	10.5
41	monooleate	($C_{18:1}$)	5	10.0
45	trioleate	($C_{18:1}$)	20	11.0
Tween				
65	tristearate	($C_{18:0}$)	20	10.5

The Polyglycolyzed Glycerides		HLB
Labrafil M-1944CS	Apricot kernel oil PEG-6 complex	
	main fatty acid: oleic ($C_{18:1}$)	3-4
Labrafil M-2125CS	Corn oil PEG-6 complex	
	main fatty acid: linoleic ($C_{18:2}$)	3-4
Labrafil WL 2609BS	Corn oil PEG-8 complex	
	main fatty acid: linoleic ($C_{18:2}$)	6-7
Labrafac CM 10	Polyglycolyzed C_8-C_{10} glycerides	
	reacted with PEG 400	10.0
Labrasol	Glyceryl caprylate / caprate and PEG-8	
	caprylate / caprate	
	main fatty acid: caprylic-capric	14.0

Address of suppliers

Aldrich, New Rd, Gillingham, Dorset, SP8 4XT.

Surfactants were kindly donated by:

Alfa Chemicals Ltd., Arc House, Binfield, Bracknell, RG42 4PZ (Labrafac, Labrasol, Labrafil series).

BASF United Kingdom Ltd., Cheadle, SK8 6QG (Cremophor RH40, Lutensol series).

Cargo Fleet Chemical Co. Ltd., Eaglescliffe, Stockton (Synperonic series).

Croda Chemicals Ltd., Snaith, Goole, DN14 9AA (Etocas, Croduret, Crill, Crillet series).

Diamond Shamrock Process Chemicals Ltd., Eccles, M30 OBH (Ethylan series).

Dow Chemical Company Ltd., Hounslow, TW5 9QY (Dowfax series).

Hoechst UK Ltd., Hounslow, TW4 6JH (Arkopal series).

Hüls (UK) Ltd., Matrix House, Milton Keynes, MK9 1NJ (Miglyol® 812).

R P Scherer Ltd., Blagrove, Swindon, SN5 8YS (Soya bean oil).

Stepan Europe., St. James Court, Warrington, WA4 6PS (Neobee M20).

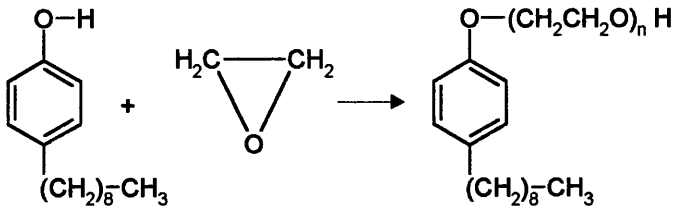
Surfachem Ltd., Thirsk Row, Leeds, LS1 4DP (Brij, Span, Tween series).

Unichema International, Bebington, Wirral, L62 4UF (Priolene).

4.2 Investigation of the inhibition of pancreatin activity by nonylphenol ethoxylated surfactants.

4.2.1 Preliminary investigations.

Nonylphenol ethoxylated surfactants (NPEs) are a class of alcohol ethoxylated surfactants formed by an alkaline catalysed reaction between ethylene oxide and the phenol hydrogen upon nonylphenol:



The ethoxy group added on may react with another molecule of ethylene oxide and so on to form a chain of varying length. The subscript n represents the ethoxylation number, which for future discussion of NPEs is taken to be identical to the number of ethoxy groups in the polyoxyethylene chain.

Preliminary investigations were necessary to establish if pancreatin activity towards a substrate of MCT was inhibited by NPEs. By increasing the degree of ethoxylation of the NPEs tested, the extent of pancreatin inhibition could then be examined in terms of the HLB value of the surfactant.

It was also necessary to establish concentrations of MCT and surfactant for use in the assay. Ideally concentrations were required which revealed the potency of inhibition of the surfactant whilst representing appropriate concentrations of surfactant and triglyceride likely to be included in a lipid-based formulation.

4.2.2 Method

The standard pH-stat assay (section 2.2) was employed to assess pancreatin activity towards MCT in the presence of NPEs. NPEs with ethoxy groups of 2, 4, 5, 6, 7, 7.5, 10, and 12 (Dowfax series) were selected for use in this study. Batches of MCT

containing NPEs at 10, 20 and 50 % $^{w}/_{w}$ were prepared for each surfactant by the premixed method. For digestions where NPEs were present at a concentration of 10 or 20 % $^{w}/_{w}$ 500 mg masses of MCT / surfactant mixture were used for each assay; this mass was increased to 2 g when the surfactant concentration was raised to 50 % $^{w}/_{w}$. Digestion profiles of pancreatin activity upon the MCT / surfactant mixtures were produced under conditions of the standard pH-stat assay, with titrant volumes recorded at intervals of five minutes over a period of one hour.

4.2.3 Results

Digestion profiles for MCT in the presence of NPEs at 10 and 20 % $^{w}/_{w}$ (with respect to MCT).

The activity of pancreatin towards MCT in the presence of NPEs at 10 % $^{w}/_{w}$ concentration (with respect to MCT) is shown in Figure 4.1. The digestion profiles are represented as line plots to aid interpretation although data was available only at 5 minute intervals. A digestion profile for the same concentration of MCT produced under identical experimental conditions is included in Figure 4.1 as a control. Figure 4.1 shows that inclusion of surfactant at a concentration of 10 % $^{w}/_{w}$ did not appear to inhibit pancreatin activity towards MCT.

Upon increasing the concentration of surfactant to 20 % $^{w}/_{w}$ (with respect to MCT) examination of the resulting digestion profiles in Figure 4.2 suggested some suppression of pancreatin activity. This is evident from comparison of the total triglyceride digested upon completion of the assay. The MCT control profile reached 90 % digestion after 60 minutes. When the nonylphenol ethoxylated surfactant with a chain of two ethoxy groups (2-NPE) was included in the reaction mixture, the total of MCT digested was reduced to 83 % over the same assay period. For the other ethoxy chain lengths tested the total percentage of MCT digested was between 83 and 90 %. There was no evidence of a reduced enzyme reaction rate at steady state due to inclusion of NPEs (Figure 4.2).

The effect of NPEs at a concentration of 20 % $^{w}/_{w}$ did not influence digestion of MCT sufficiently to demonstrate definite inhibition of enzyme activity or to enable examination of a relationship between pancreatin activity and ethoxy chain length of the surfactant.

Digestion profiles for MCT in the presence of NPEs at 50 % $^{w}/_{w}$ (with respect to MCT).

The digestion profiles of MCT in the presence of NPEs at 50 % $^{w}/_{w}$ are illustrated in Figure 4.3a (ethoxy chain lengths 0 to 6) and Figure 4.3b (ethoxy chain lengths 6 to 12). A control profile of MCT (1 g) digested under identical conditions is included in Figure 4.3 to represent pancreatin activity in an uninhibited system.

All digestion profiles containing NPEs at 50 % $^{w}/_{w}$ (with respect to MCT) showed suppression of pancreatin activity compared to the control MCT profile (Figure 4.3 a-b). NPEs with ethoxy chain lengths from 4 to 7.5 suppressed pancreatin activity, as shown by the decreased percentage of MCT digested at the end of the assay compared to the control. However the shape of these profiles were similar to the MCT control profile (Figure 4.3 a-b), with no evidence of a lag phase. For 10-NPE a lag phase occurred at the beginning of the profile followed by a surge in enzyme activity; the same pattern in a more pronounced form was shown in the presence of 12-NPE (Figure 4.3b).

Figure 4.3a includes a digestion profile for MCT in the presence of nonylphenol. This profile demonstrated that extensive suppression of MCT digestion can be caused by the nonylphenol moiety without ethoxylation. The highly lipophilic 2-NPE (Figure 4.3a) exhibited a pattern of enzyme suppression similar in character to nonylphenol however inhibition was partially overcome 35 minutes into the assay.

Overall the inclusion of 50 % $^{w}/_{w}$ surfactant (with respect to MCT) in the assay system suggested the existence of a relationship between the activity of pancreatin towards MCT and the degree of surfactant ethoxylation. This in turn inferred a possible correlation between the potency of inhibition of a surfactant and its HLB value.

Figures 4.3 a-b also demonstrated that the quantity and ratio of MCT (1 g) to surfactant (1 g) used within the assay was sufficient to reveal varying extents of inhibitory activity from the surfactants tested. Therefore for all future digestions performed in Chapter 4, MCT to surfactant concentration was maintained at 50 % $^{w}/_{w}$ (with respect to MCT) using a mixture of 1 g of MCT and 1 g of surfactant in each case. These quantities are also of a level likely to be found in a lipid-based formulation for oral administration.

4.3 Investigation of the relationship between inhibition of pancreatin activity by NPEs and surfactant HLB value.

Investigations in section 4.2 had indicated an apparent relationship between pancreatin activity towards MCT and the degree of ethoxylation of NPEs. However only a limited range of NPEs had been examined covering ethoxy chain lengths from 2 to 12. In order to examine this suspected relationship further, an additional twenty NPEs from various manufacturers (listed in section 4.1.6) were obtained with ethoxy chain lengths from two to thirty. This enabled the potency of inhibition of NPEs to be examined across a wide range of HLB values.

4.3.1 Method

Digestion profiles of MCT were produced in the presence of each surfactant to assess the extent of pancreatin activity. Each digestion involved 1 g of MCT and 1 g of nonylphenol ethoxylated surfactant, premixed prior to digestion under conditions of the standard pH-stat assay. Titrant volume was recorded every 5 minutes over the 60 minute assay.

Due to long delays in obtaining some of the NPEs the experimental work for this investigation was divided into two stages. The surfactants readily available were used early in the study (Stage A of the study). At a later date digestion profiles were produced using the remaining NPEs (Stage B of the study).

4.3.2 Results

<u>Stage A</u>

The digestion profiles in Stage A of the investigation were produced using NPEs with HLB values ranging from 0 to 17. The effect of the NPEs upon pancreatin activity was seen to vary greatly. This is demonstrated by comparison of the amount of triglyceride digested five minutes after commencement of the assay (between 0.5 to 26 % of the total MCT present), and upon completion of the sixty minute assay (between 13 to 80 % of the total MCT present).

Due to the large number of surfactants tested, a method other than direct comparison of digestion profiles was required to allow examination of the relationship between pancreatin activity and surfactant HLB value. The parameter selected for this purpose was the time taken for pancreatin to convert a specified percentage of triglyceride ($t_{\%}$) to monoglyceride and two fatty acids.

The $t_{\%}$ value does not represent a fundamental kinetic parameter of pancreatin activity or a particular inhibitory mechanism but is used solely to compare the efficiency of pancreatin digestion of MCT within the standard pH-stat assay. A general parameter of this type was required due to the wide variation in levels of inhibition. $t_{\%}$ allowed the relationship between inhibition of lipolysis and surfactant HLB value to be examined for a large number of surfactants in a manner independent of complications involved when comparing digestion profiles of a non uniform shape.

As the extent of digestion differed considerably between profiles, it was necessary to compare various $t_{\%}$ values. A range of $t_{\%}$ values (2.5, 5, 10, 25 and 50 %) were therefore calculated from each digestion profile to enable determination of the $t_{\%}$ value common to most profiles. Calculation of $t_{\%}$ was achieved by linear interpolation between the two data points either side of the required percentage of digestion on the profile.

In combination the $t_{10\,\%}$ and $t_{25\,\%}$ parameters were found to represent the complete range of NPEs tested in Stage A and provided a means of graphical presentation of the inhibitory properties of surfactants towards pancreatin. High $t_{\%}$ values indicated potent inhibitors as pancreatin activity was depressed whereas low $t_{\%}$ values represented rapid enzyme digestion of MCT and thus surfactants with weak inhibitory properties.

Figure 4.4 illustrates the relationship between the selected $t_{\%}$ parameter and surfactant HLB value for NPEs tested in Stage A. For surfactants with HLB values up to eleven $t_{25\,\%}$ decreased towards a minimum at HLB 11 (Figure 4.4). This suggested a loss of potency of inhibition with decreased surfactant lipophilicity. Between HLB values of 12 to 15 a sharp rise in $t_{10\,\%}$ and $t_{25\,\%}$ can be seen as surfactants become more inhibitory towards pancreatin reaching an apparent maximum inhibitory HLB value of approximately 15 (Figure 4.4). With HLB values of 16 and above inhibition appeared

to decline although only three surfactants were available for testing in this range (Figure 4.4).

Stage B

Upon receipt of the remaining surfactants required for the study digestion profiles were produced in the same manner as for Stage A. For some profiles in Stage A, a high percentage of MCT had been digested when the first titrant volume was recorded 5 minutes after the start of the assay. Frequency of titrant volume recordings were thus increased in Stage B to improve accuracy of the linear interpolation. Titrant volumes were recorded every minute for the first twenty minutes of the assay followed by every 5 minutes for a further forty minutes.

Calculation of $t_{\%}$ (2.5, 5, 10, 25 and 50 %) was made from each digestion profile, where data allowed, in the same manner as described in Stage A. Examination of the resulting values revealed $t_{2.5\,\%}$ and $t_{5\,\%}$ to represent the complete range of NPEs tested in Stage B and these are shown in Figure 4.5 as a function of the HLB value of the surfactant. Upon examination of the relationship between $t_{\%}$ and HLB value (Figure 4.5) the same trend was seen as already described for the surfactants tested in Stage A (Figure 4.4).

The sharp increase in potency of inhibition seen for NPEs with HLB values from 10 to 15 in Figure 4.5 was examined further by means of a log / linear plot. Figure 4.6 demonstrates an exponential relationship between $t_{2.5\,\%}$ and the HLB value of NPEs. This indicates a monotonic increase in the potency of inhibition of NPEs according to the number of ethoxy groups present, when in the HLB range of 10 to 15.

Comparison of results from Stage A and B of the investigation revealed NPEs with similar HLB values to have a greater inhibitory effect upon pancreatin activity in Stage B than when tested in Stage A. This effect is illustrated in Figure 4.7 which demonstrates that digestion on profiles from Stage B was slower to proceed, hence values of $t_{25\,\%}$ are increased when compared to a surfactant with the same HLB value used in Stage A. Control experiments to recheck operation of the pH-stat, stability of the assay components and NPEs revealed the explanation for this behaviour involved the batch of pancreatin used for the digestion.

4.4 Investigation of the susceptibility of pancreatin to the inhibitory properties of nonylphenol ethoxylated surfactants.

4.4.1 Introduction

The difference in susceptibility of pancreatin to the inhibitory properties of NPEs with the same HLB value appeared to be related to the batch of pancreatin used for the digestion (section 4.3.2). Batch I used in Stage A being less susceptible to inhibition mediated by NPEs than batch II used in Stage B. However only the magnitude of the inhibitory effect was altered, with the relationship between $t_{\%}$ and surfactant HLB value remaining the same (Figures 4.4 / 4.5).

When the activity of batch I and II of pancreatin had been assessed using a substrate of tributyrin (section 2.3), both batches had been observed to have a similar rate of hydrolytic activity. Likewise an investigation in Chapter 3 had not demonstrated conclusively that batch I was more active towards MCT in the presence of Cremophor than batch II.

Although repeatability of results had been demonstrated when using the same batch of pancreatin to digest MCT / nonylphenol ethoxylated surfactant mixtures, repeatability had not been examined between batches. An investigation was thus designed to examine further the inhibition of pancreatin by NPEs to ensure conclusions from the inhibition study were not specific to a particular batch of pancreatin.

The surfactants chosen for the investigation were 10-NPE and 12-NPE (Dowfax series). These were selected as they had HLB values in the region where, in Figure 4.7, the difference in enzymatic activity between the two batches was most pronounced. In addition each batch of pancreatin was also used to produce a digestion profile of MCT. This was to ensure any difference in pancreatin activity was due solely to inclusion of NPEs in the reaction mixture.

4.4.2 Method

Digestion profiles using 1 g of MCT as substrate were produced to determine the activity of batch I and II of pancreatin in an uninhibited system. In addition batch IIa

and batch IV of pancreatin were included in this study for the same reasons as stated in Chapter 3. Digestion profiles were also produced using the same batches of pancreatin acting upon a substrate of MCT (1 g) premixed with 1 g of surfactant (10 or 12-NPE).

All digestion profiles were produced under conditions of the standard pH-stat assay. Titrant volume was recorded every minute for the first twenty minutes followed by every 5 minutes for the remainder of the one hour assay. In the case of batch I pancreatin, due to lack of enzyme, data from Stage A of the investigation was included for comparison. In this case titrant volumes had been recorded at 5 minute intervals over the one hour assay period.

4.4.3 Results

The activity of each batch of pancreatin towards 1 g of MCT is shown in Figure 4.8. Little difference was seen between the profiles from batches I, II and IIa although batch I appeared to have slightly higher activity towards the substrate (Figure 4.8). Batch IV of pancreatin had the highest rate of enzyme activity towards MCT (Figure 4.8) as expected from the higher standard of minimum activity stated by the manufacturer. No difference was seen between the batches of pancreatin in terms of total triglyceride digested at the end of the assay which varied between 82 to 84 %. Figures 4.9 and 4.10 illustrate how the presence of 10 and 12-NPE respectively caused an alteration in enzyme activity towards MCT, the magnitude of which varied according to the batch of pancreatin used. When compared with the virtually identical activity observed between batches when digesting MCT alone, (Figure 4.8) inclusion of surfactant revealed the enhanced ability of certain batches of pancreatin to overcome inhibitory effects.

In the presence of surfactant, digestion profiles from all batches of pancreatin exhibited a lag phase (which was considerable for batch II pancreatin) followed by a period of enzyme activity at steady state (Figures 4.9-4.10). The rate of enzyme activity at steady state appeared to be diminished compared to the rate shown on the uninhibited MCT profiles (Figure 4.8). To quantify this change in pancreatin activity

use was made of the parameter $V_{\%}$, described in Chapter 3 (Equation 3.2) as the degree of inhibition.

$$V_{\%} = \frac{Vi}{Vo} \times 100 \qquad \text{Equation 3.2}$$

where

Vo = Reaction rate shown by pancreatin at steady state towards 1 g of MCT.

Vi = Reaction rate shown by the same batch of pancreatin at steady state towards MCT (1 g) in presence of nonylphenol ethoxylated surfactant (1 g).

$V_{\%}$ was calculated using Equation 3.2 to describe enzyme reaction rate at steady state in the presence of NPEs as a percentage of the uninhibited rate shown towards MCT. The enzyme reaction rate towards MCT, Vo, was determined from the linear portion of each digestion profile in Figure 4.8 by means of linear regression. Calculation of enzyme reaction rate in the presence of 10-NPE, $Vi_{\text{10-NPE}}$, and 12-NPE, $Vi_{\text{12-NPE}}$, was determined in the same manner as for Vo from profiles in Figure 4.9 and Figure 4.10 respectively, where data allowed. The resulting values are shown in Table 4.1 together with the $V_{\%}$ values calculated via Equation 3.2.

Batch of pancreatin	Vo % digested min^{-1}	$Vi_{\text{10-NPE}}$ % digested min^{-1}	$V_{\%}$ for 10-NPE	$Vi_{\text{12-NPE}}$ % digested min^{-1}	$V_{\%}$ for 12-NPE
I	5.94	3.84	65	3.19	54
II	4.86	3.17	65	2.62	54
IIa	4.90	3.27	67	-	-
IV	8.31	6.77	81	4.47	54

Table 4.1 Comparison of reaction rates shown at steady state by each batch of pancreatin towards a substrate of MCT in the presence and absence of nonylphenol ethoxylated surfactants.

In all cases $V_{\%}$ confirmed that the presence of NPEs decreased enzyme activity at steady state compared to the activity shown by the same batch of pancreatin towards MCT alone (Table 4.1). All batches of pancreatin tested showed a $V_{\%}$ of 54 % in the presence of 12-NPE, therefore enzyme activity towards the same substrate had been reduced by 46 % irrespective of the inherent activity of the pancreatin used (Table

4.1). Considered in isolation this result suggested $V_{\%}$ to be a constant value relative to the surfactant used. However when results from the less inhibitory surfactant, 10-NPE were examined, an increased value of $V_{\%}$ was shown by batch IV of pancreatin (Table 4.1). Further data was therefore required to examine if $V_{\%}$ was of a variable or constant nature with respect to the degree of ethoxylation of a surfactant.

4.4.4 Relationship between the decrease in pancreatin activity at steady state ($V_{\%}$) and the degree of ethoxylation of NPEs.

Introduction

The presence of surfactant was noted in section 4.4.3 to reduce enzyme activity at steady state (represented by $V_{\%}$), however it was not known if the extent of this reduction was related to the degree of ethoxylation of the surfactant and / or the batch of pancreatin used. To examine further the influence of the degree of ethoxylation of NPEs on the activity of pancreatin (batch I and II) in terms of $V_{\%}$, a study was designed which involved fifteen NPEs with ethoxylation numbers from 0 to 30 (Dowfax and Arkopal series).

Digestion profiles were produced in the presence of each surfactant in same manner as described for Stage B (section 4.3.2) using batch II of pancreatin. Profiles had already been obtained in the presence of the same surfactants using batch I of pancreatin in Stage A. This enabled comparison of activity between the two batches in the presence of the same surfactant.

Results

When pancreatin activity was quantified at steady state using Equation 3.2, $V_{\%}$ values for batch I and II of pancreatin did not appear to differ in the presence of the same surfactant. However $V_{\%}$ was noted to vary with the degree of ethoxylation of the surfactant. This variation in $V_{\%}$ is shown in Table 4.2 in terms of the length of the ethoxy chain and corresponding HLB value of the surfactant tested.

Surfactant		Range of $V_{\%}$
Ethoxy chain length	HLB value	
0	0	44-50
2	5.7	70-83
4-7	8.9-11.7	78-100
7.5-8	12-12.3	70-80
10-11	13.3-13.8	61-66
12	14.1	54
30	17.1	38-40

Table 4.2 Relationship between pancreatin activity at steady state, ($V_{\%}$) and the degree of ethoxylation of the surfactant present.

The relationship shown in Table 4.2 between the percent of the uninhibited reaction rate remaining in the presence of surfactant, (represented by $V_{\%}$) and the HLB value of the surfactant reflects the trend seen in Figure 4.4. For surfactants with a HLB value of 0 to 11 $V_{\%}$ slowly increased representing decreasing inhibitory effect. $V_{\%}$ fell in value for surfactants with HLB values between 12 and 14, indicating increased inhibition of pancreatin activity. The highly ethoxylated surfactant with a HLB of 17 also showed extensive inhibition of enzyme activity at steady state. However the severe inhibition caused by surfactants with HLB values between 14 and 16 is not reflected in Table 4.2 as these surfactants extended the lag phase to such an extent that pancreatin did not reach steady state within the one hour assay.

In summary the parameter of $V_{\%}$ has shown NPEs to cause a reduction in enzyme activity at steady state. The results suggest the extent of this reduction is related to the degree of ethoxylation of the surfactant and appears unaffected by the batch of pancreatin used.

The duration of the lag phase on each digestion profile used to obtain $V_{\%}$ was measured and is illustrated in Figure 4.11 as a function of the HLB value of the surfactant present. From a HLB of 10 profiles produced using batch II pancreatin exhibited a longer lag phase than profiles produced using batch I pancreatin (Figure 4.11); the extent of this difference increased from a HLB value of 10 up to 15. Inhibition of pancreatin activity by NPEs therefore appears to be mediated mainly by an extension of the lag phase with some reduction in enzyme reaction rate at steady state. For both the lag phase and $V_{\%}$ the extent of the inhibition is related to the

degree of ethoxylation of the surfactant. Results suggest the inter-batch variation in susceptibility of pancreatin to inhibitory effects of NPEs is related only to a reaction occurring during the lag phase. #

4.4.5 Discussion regarding the variation in susceptibility of pancreatin to inhibitory effects of alcohol ethoxylated surfactants.

The difference in susceptibility of various batches of pancreatin to the inhibitory effects of alcohol ethoxylated surfactants appears related to the initial stages of triglyceride digestion. Interestingly enzyme activity at steady state, although reduced in the presence of surfactants, was virtually unchanged between batches of pancreatin. When pancreatin acts upon a substrate of MCT under conditions of the standard pH-stat assay enzyme activity reaches steady state immediately. This occurs despite the supramicellar concentration of bile salt in the simulated bile solution which would be expected to inactivate lipase by causing displacement of the enzyme from the lipid interface[173]. However colipase present in pancreatin may overcome any desorbing activity of bile salts[173] resulting in immediate lipase activity, possibly by acting as an anchor for lipase to bind to the substrate[174].

The process of colipase binding to the lipid substrate has been suggested to occur via hydrogen bonding with ester linkages in the lipid substrate[20]. The hydrogen bonds have been reported to be easily disrupted by non-ionic surfactants[20] and octylphenol ethoxylate has been demonstrated to displace colipase from the lipid substrate interface[173]. Tensioactive agents, such as non-ionic surfactants, have also been stated to mediate inhibition of lipase by affecting the interfacial quality of the substrate[175].

In the work presented here pancreatin has demonstrated the ability to overcome the initial lag phase caused by the presence of alcohol ethoxylated surfactants or Cremophor (section 3.4), although the time taken to reach steady state did vary. This initial inhibition of enzyme activity upon commencement of the assay may be a direct

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#Susceptibility of pancreatin to the inhibitory properties of linear alcohol ethoxylates.
Digestion profiles of MCT produced in the presence of LAEs (Lutensol AO series) were also performed using batch I and II of pancreatin. The resulting profiles from batch II exhibited an extended lag phase compared to profiles including the same surfactant digested by batch I. This indicates that batch I and II of pancreatin exhibit different activity regardless of the type of alcohol ethoxylated surfactant present.

result of surfactant altering the quality of the substrate interface. Colipase, whilst able to overcome any desorption of lipase due to bile salts in the reaction milieu, may be unable to immediately overcome effects from the surfactant molecules.

Surfactant molecules could initially be assumed to accumulate at the lipid interface blocking adsorption of colipase, possibly by steric hindrance or interference with hydrogen bonding between colipase and triglyceride substrate. Eventually binding of some colipase would allow limited lipolysis to proceed. The resulting accumulation of lipolytic products at the interface would thus promote further colipase binding and increase enzymatic activity to steady state; this suggests the colipase partitioning theory already proposed for Cremophor also applies to inhibition mediated by other non-ionic surfactant types.

Studies by other workers gives support to the above hypothesis that colipase rather than lipase is responsible for overcoming inhibition from non-ionic surfactants. Colipase has been shown to activate lipase in the presence of substrate interfaces to which lipase cannot bind alone[176]. The addition of colipase in increasing amounts to a detergent inhibited system has also been demonstrated to eventually abolish the lag phase[177].

If the ability of colipase to restore lipase activity is related to length of the lag phase, the duration of the lag phase must reflect efficiency of the colipase. For this study using MCT / nonylphenol ethoxylated surfactant mixtures digestion profiles from batch I pancreatin had a shorter lag phase compared to batch II (Figure 4.11). This infers that batch I pancreatin contains colipase with higher activity than batch II.

The literature goes some way to supporting this assumption as the structure of colipase has been found to be related to the duration of the lag phase. Colipase isolated from porcine pancreatic glands can vary in the number of amino-acid residues present in the peptide chain. Proteolysis at the N-terminal will result in conversion of procolipase (colipase-101) to the more active colipase-96 form[20]. When acting upon a substrate of phosphatidylcholine stabilized triglyceride emulsion colipase-96 can overcome lag times at 100 times lower concentrations than procolipase[178]. In addition cleavage of colipase-96 at the C-terminal end to colipase-85 reduced lag time further by a factor of six[23].

Several factors could be responsible for the difference in duration of the lag phase between batch I and II pancreatin if colipase is proposed to be the cause. Both batches could contain the same form or forms of colipase in identical proportions however the concentration of colipase in the bulk enzyme extract may be different. Alternately the form of colipase may be different due to the crude nature of the material rendering batch I more efficient at adsorption to the lipid interface in the presence of non-ionic surfactants. Unfortunately no information was available from the manufacturer or literature regarding the possible form or concentration of colipase present in the pancreatin used in this series of experiments.

No discernible difference in activity was evident between batch I and II of pancreatin when acting upon MCT in the absence of non-ionic surfactants. This tends to suggest the concentrations or proportions of colipase present in each batch were adequate to overcome any influence upon enzyme binding from bile salts or lecithin. The difference between batch I and II of pancreatin only became apparent when the interface was affected by surfactant molecules to such a degree as to make binding of colipase difficult.

Further work to investigate this phenomenon could include the use of pure lipase in the assay system with subsequent addition of known forms of colipase to the reaction mixture. This would establish which form of colipase is particularly efficient at overcoming the lag phase. Another approach could involve increasing the concentration of pancreatin used in the assay. This may establish if differences in the ability to overcome the lag phase between batches was solely related to concentration of colipase in the bulk extract, rather than the form of colipase present.

As the simulated bile solution used in the standard pH-stat assay contained calcium, upon addition of trypsin any colipase present should be cleaved to the more efficient colipase-85[23]. If extent of colipase cleavage is the limiting factor this approach should result in lag phases of the same duration for both batches of pancreatin used.

4.5 A hypothesis to explain the relationship between the HLB value of NPEs and the extent of inhibition of pancreatin activity.

NPEs have an inhibitory effect on the activity of pancreatin towards MCT. The extent of the inhibition appears to be related to the HLB value of the surfactant. The same relationship between HLB value of NPEs and potency of inhibition towards pancreatin activity was demonstrated by the various parameters of $t_{\%}$ (Figures 4.4 & 4.5), duration of the lag phase (Figure 4.11), and $V_{\%}$ (Table 4.2).
Examination of $t_{\%}$ revealed that within a HLB range of 10 to 15 (ethoxylation number of 5 to 15) the potency of inhibition of NPEs increased exponentially with the extent of surfactant ethoxylation (Figure 4.6). Surfactants with a HLB value of approximately 15 had maximum inhibitory effect represented by a lag phase which lasted for the duration of the assay, however as illustrated in Figure 4.10 these long lag periods can be overcome if the assay period is extended. As the degree of ethoxylation of the surfactant, and hence the hydrophilic character is increased above a HLB of 15 the potency of inhibition shown by the surfactant declines.
Digestion profiles produced in the presence of NPEs with dominant lipophilic character (ethoxy chain lengths < 5) showed suppression of enzyme reaction rate at steady state without evidence of a lag phase. Digestion profiles with nonylphenol present also inhibited pancreatin by reduction of enzyme reaction rate with no lag phase present. A further characteristic of profiles produced in the presence of lipophilic surfactants was a reduction in the amount of MCT digested at end of the assay compared to the MCT control.

From the above discussion alteration in the activity of pancreatin by NPEs appears related to the degree of ethoxylation of the surfactant and hence surfactant hydrophile-lipophile balance. This may perhaps be explained by consideration of the behaviour of NPEs at a lipid-water interface. The orientation of nonylphenol ethoxylated surfactant molecules at an oil-water interface has been suggested to vary depending upon length of the ethoxy chain, as represented in Figure 4.12 below[170].

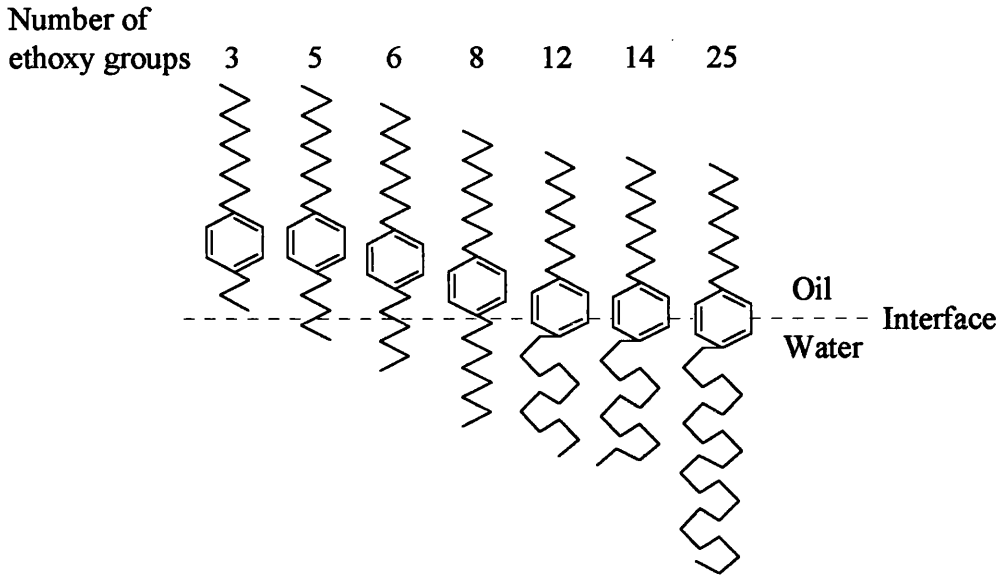


Figure 4.12 The state of orientation of NPEs with different numbers of ethoxy groups at an oil-water interface. (Reproduced from reference[170].)

For NPEs with high lipophilic character the surfactant molecule is represented as completely partitioned into the oil phase (Figure 4.12). As the number of ethoxy groups in the chain is increased from 5 through to 8 the hydrophilic ethoxy chain gradually penetrates into the aqueous phase until only the nonylphenol moiety remains in the oil. Additional ethoxylation after this point results in the ethoxy chain adopting a meander form in the water with the hydrocarbon group also penetrating further into the aqueous phase.

In the standard pH-stat assay triglyceride droplets, probably with associated lecithin, are dispersed in an aqueous reaction mixture as an oil in water emulsion. The nonylphenol ethoxylated surfactant molecules are assumed to be located mainly at the interface between the triglyceride droplet and the aqueous environment. If the surfactant molecules adopt a similar conformation as illustrated in Figure 4.12 according to the length of their ethoxy chain, a mechanism to explain the change in their inhibitory effects with HLB can be proposed.

Nonylphenol and NPEs with ethoxy chain lengths below 5 have dominant lipophilic character. From Figure 4.12 the highly lipophilic nature of these surfactants would result in the complete molecule being partitioned into the triglyceride droplet. The lipase and colipase would still be able to bind and start digestion immediately as

demonstrated by the lack of a lag phase at the beginning of the digestion profiles produced in the presence of these surfactants. Penetration of the surfactant completely into the oil may cause a substrate dilution effect by acting as inert spacers, resulting in less triglyceride available at the interface for lipase to act upon. This is analogous to the concept of substrate dilution used in lipolytic enzyme kinetic studies where a lipid or detergent molecule known not to be a substrate for the enzyme is introduced into the system[179].

The digestion profiles support the substrate dilution theory by exhibiting decreased enzyme activity at steady state and a reduction in the extent of triglyceride digestion achieved within the assay period. In addition little difference was seen between digestion profiles produced in the presence of these surfactants when using different batches of pancreatin. This could be explained by assuming binding of colipase to substrate experiences little disruption as some triglyceride is still immediately available, therefore colipase with higher activity is not necessary.

For NPEs with chains containing from 5 to 15 ethoxy groups the potency of inhibition increases with the degree of ethoxylation of the surfactant. Chains composed of 6 to 8 ethoxy groups will start to form an oxyethylene mantle around the lipid droplet restricting access of colipase to the interface and thus delaying enzyme adsorption. As the ethoxy chain is increased to 9 groups and above the mantle will become harder to penetrate as the ethoxy chain adopts a meander form. This accounts well for the increase in duration of the lag phase with the degree of ethoxylation of the surfactant, reflecting restricted colipase binding to substrate. It also illustrates the dependence of lipase activity on the ability of colipase to overcome the presence of surfactant, thus revealing the advantage of colipase with higher activity.

The potency of inhibition of NPEs with ethoxy chain lengths of above 15 starts to diminish as hydrophilic character of the surfactant is increased by lengthening of the ethoxy chain. Figure 4.12 illustrates how partitioning of the nonylphenol moiety further into the aqueous reaction system is likely due to the dominant hydrophilic character of the molecule. These highly hydrophilic surfactants may show reduced inhibition due to preferential partitioning of the surfactant from the lipid interface into the aqueous reaction medium. This would result in enhanced colipase binding to the lipid interface and a subsequent increase in the rate and extent of MCT lipolysis.

4.6 Investigation to determine the effect of various types of non-ionic surfactants on pancreatin activity towards MCT.

The aim of this investigation was twofold, to determine if the relationship between HLB value and the potency of inhibition demonstrated by NPEs held for other non-ionic surfactants suitable for use in pharmaceutical formulations. Secondly to examine for a relationship between the potency of inhibition and chemical structure of surfactants to gain further information regarding the mechanism of inhibition. Although alcohol ethoxylated surfactants are not suitable for oral administration the linear alcohol ethoxylated surfactants (LAEs) were included in this study for comparison with results from the NPEs due to simplicity of their chemical structure. The potency of inhibition of LAEs was examined in relation to HLB value and the length of the alcohol derived linear alkyl chain (section 4.7).
The castor oil ethoxylates, sorbitan esters, polyethoxylated sorbitan esters and polyglycolized glycerides were selected as examples of surfactant types which might be considered for pharmaceutical use. These groups of surfactants covered a wide range of HLB values however the number of surfactants available in each group was limited. Each group of pharmaceutically acceptable surfactants is discussed individually in terms of inhibition of pancreatin activity in relation to their chemical structure and HLB values in sections 4.8 to 4.10.

The following method was used to assess pancreatin activity towards MCT in the presence of each surfactant in turn (listed according to type in section 4.1.6). A digestion profile was produced for each surfactant under conditions of the standard pH-stat assay, with 1 g of MCT and 1 g of surfactant used for each digestion. The titrant volume was recorded every minute for the first 20 minutes, followed by recordings at 5 minute intervals for the remaining 40 minutes of the assay. Batch II pancreatin was used for all digestions performed. The resulting profiles are discussed under the relevant surfactant group heading.

4.7 Linear alcohol ethoxylated surfactants.

The purpose of this study was to examine the ability of linear alcohol ethoxylates (LAEs) to inhibit pancreatin activity towards MCT. Any inhibitory effect observed due to LAEs was intended for comparison with results already obtained from the NPEs. Nonylphenol had been demonstrated in section 4.2.3 to have an inhibitory effect upon pancreatin therefore the inhibition mediated by NPEs may also be due in part to influence from the nonylphenol moiety. As the linear alcohol ethoxylates do not possess an aromatic moiety, any inhibitory activity observed could be attributed only to the linear alkyl chain and / or the ethoxy chain.

LAEs are generally made by addition of ethylene oxide to alcohol using KOH catalysis. Formation of the ethoxylate occurs in the same manner as for the NPEs, with a reaction between the hydrogen of the alcohol group and ethylene oxide. The final product of ethoxylation is represented by the following formula:

$$CH_3(CH_2)_x\,(CH_2CH_2O)_nH$$

where $x + 1$ = The number of carbons present in the linear alkyl chain.

n = The ethoxylation number.

Two main series of LAEs were obtained for testing, the Lutensol AO series (C_{13}-C_{15} alkyl chain) and the Brij series. The alcohol moiety of the Lutensol AO series is produced synthetically by the oxo process; a method which converts olefin hydrocarbon vapors into aldehyde followed by hydrogenation to alcohol. The Brij and Lutensol AT series (mixed $C_{16\text{-}18}$ alkyl chain) are described as comprised of fatty alcohols, obtained from natural fats and wax containing substances.

The formulae of the alcohols used for the alkyl chain of the Brij surfactants are listed below:

Common name	Structural Formula	Referred to in text as:
lauryl alcohol	$CH_3(CH_2)_{10}CH_2OH$	($C_{12:0}$)
cetyl alcohol	$CH_3(CH_2)_{14}CH_2OH$	($C_{16:0}$)
stearyl alcohol	$CH_3(CH_2)_{16}CH_2OH$	($C_{18:0}$)
oleyl alcohol	$CH_3(CH_2)_7CH{=}CH(CH_2)_7CH_2OH$	($C_{18:1}$)

LAEs with ethoxylation numbers of 3, 8, 10, 12 (Lutensol AO series), 11 (Lutensol AT series) and 2, 4, 10, 20 and 23 (Brij series) were tested for inhibitory activity towards pancreatin by production of digestion profiles as described in section 4.6.

4.7.1 Ability of LAEs to inhibit pancreatin.

The digestion profiles produced using the Brij and Lutensol AO series of LAEs are shown in Figures 4.13 and 4.14 respectively, with the ethoxylation number and HLB value indicated for each surfactant. Comparison of the control MCT profile with digestion profiles produced in the presence of LAEs revealed all surfactants tested to have an inhibitory effect upon pancreatin activity (Figure 4.13 and 4.14).
The rate of MCT digestion during the first 20 minutes of the assay decreased as the number of ethoxy groups increased from 2 to 10 inclusively (HLB 4.9 to 13.5) (Figure 4.13 and 4.14). For LAEs with > 10 ethoxy groups (HLB of 14.3 and above) the inhibitory effect of LAEs on pancreatin activity lessened (Figure 4.13 and 4.14).

4.7.2 Comparison of the potency of inhibition of LAEs and NPEs in terms of the ethoxylation number of the surfactants.

To compare the potency of inhibition shown by LAEs and NPEs towards pancreatin, digestion profiles were grouped according to the ethoxylation number of the surfactants. Digestion profiles from surfactants with the same number of ethoxy groups (2, 4, 10 to 12 and 20 to 23) are shown in Figures 4.15 a-d.

All alcohol ethoxylated surfactants with 2 ethoxy groups (Figure 4.15a) caused a similar decrease in rate and final extent of MCT digestion compared to the control, irrespective of the alcohol moiety present. A difference was noted in the shape of the digestion profiles as a smooth curve was observed in the presence of LAEs, as opposed to the two stage digestion profile shown by 2-NPE (Figure 4.15a).
Figure 4.15b revealed higher inhibitory activity towards pancreatin from a LAE with an ethoxylation number of 4 (4-LAE) compared to 4-NPE. The 4-LAE inhibited pancreatin to almost the same extent as 7-NPE (Figure 4.15b). Likewise the profiles produced in the presence of 10-LAE also showed greater inhibition of pancreatin activity than 10-NPE (Figure 4.15c). In the case of 10-$LAE_{16:0}$ the extent of pancreatin inhibition was equivalent to that shown by the 12-NPE profile (Figure 4.15c). Comparison of digestion profiles produced in the presence of 3 and 8-LAE also exhibited higher inhibitory activity towards pancreatin than NPEs with the same number of ethoxy groups (results not shown).
LAEs with ethoxylation numbers from 3 up to 10 inclusively therefore appear to be more efficient inhibitors of pancreatin activity when compared to NPEs with identical ethoxylation numbers. However in the case of the highly ethoxylated 20 and 23-LAE surfactants with HLB values of > 15 the reverse situation is seen, with the LAEs having a reduced inhibitory effect compared to 20 and 23-NPE (Figure 4.15d). This effect also occurred for a LAE with an ethoxylation number of 12 (HLB of 14.3) which exhibited less inhibition than 12-NPE (Figure 4.15c).

4.7.3 Comparison of the potency of inhibition of LAEs and NPEs in relation to surfactant HLB value.

To demonstrate the potency of inhibition of LAEs and NPEs in terms of HLB value, the time taken for pancreatin to digest 2.5 % of MCT, $t_{2.5\,\%}$ for both groups of surfactants is shown in Figure 4.16. A relationship can be seen between surfactant HLB value and $t_{2.5\,\%}$ for the LAEs which closely resembles the general trend shown by the NPEs (described in section 4.3.2).
For the LAEs with HLB values in the range of 10 to 13 inhibition increases steeply (Figure 4.16). The inhibitory effect reaches an apparent maximum at a HLB value of

13 (corresponding to an 11-LAE) compared to the apparent maximum value of 15 shown by NPEs (corresponding to a 15-NPE). Figure 4.16 also reveals a sharp reduction in inhibition as the HLB increases above 13 for LAEs which follows the pattern shown by NPEs, although for LAEs the decrease starts at a lower HLB value.

4.7.4 Influence of the alkyl chain of LAEs on inhibition of pancreatin activity.

Digestion profiles in Figures 4.15a-d were examined to determine if the length of the linear alkyl chain influenced the extent of inhibition of pancreatin by LAEs. Comparisons were made between digestion profiles from surfactants with alkyl chains of $C_{12:0}$ $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ (Brij series), a C_{16-18} mixture (Lutensol AT series), and a C_{13-15} mixture (Lutensol AO series).

The $C_{16:0}$ surfactants with 2 and 10 ethoxy groups had slightly increased inhibitory behaviour compared to the $C_{18:0}$ and $C_{18:1}$ chains with the same ethoxylation number (Figures 4.15a and c). However for highly ethoxylated LAEs the reverse situation was seen, as the 20-$LAE_{16:0}$ exhibited less inhibition of pancreatin than the C_{18} chain equivalents (Figures 4.15d).

Investigation of the effect of a mixed C_{16-18} chain (11-LAE_{16-18}) (Figure 4.15c) could not be determined as another 11-LAE was not available for comparison. Likewise no conclusion could be drawn regarding a difference in inhibitory behaviour between the fatty alcohol derived hydrocarbon chains ($C_{16:0}$, $C_{18:0}$, $C_{18:1}$, C_{16-18}) and the synthetic alcohol derived chain (C_{13-15}) due to the small number of C_{13-15} surfactants available. However a direct comparison was possible for the surfactants with an ethoxy chain length of 10, where less inhibition was seen from the C_{13-15} than the longer alkyl chain equivalents (Figure 4.15c). The existence of a double bond in the $C_{18:1}$ chain appears to confer an increase in potency of inhibition when compared to the saturated $C_{18:0}$ chain with the same extent of ethoxylation in Figures 4.15a, c and d.

4.7.5 Mechanism proposed to explain the inhibitory effect of LAEs on pancreatin activity.

The mechanism of inhibition proposed to explain the effect of NPEs on pancreatin activity involved the position of the lipophilic and hydrophilic moieties of the surfactant when partitioned into the lipid interface. If orientation of the linear alcohol ethoxylated surfactant molecule at the interface is also related to potency of inhibition towards pancreatin, results from this study can possibly be explained.

The LAEs exhibited higher inhibitory activity towards pancreatin than NPEs with the same ethoxylation number up to a HLB of approximately 13, thereafter as surfactant hydrophilic character was increased the NPEs had higher inhibitory activity. The difference in inhibitory activity between the two groups of alcohol ethoxylated surfactants may relate to the partitioning behaviour of the hydrocarbon moiety of the surfactant into the lipid interface.

The linear alkyl chain of LAEs has higher hydrophobic character than the nonylphenol moiety of NPEs and could therefore be assumed to provide the most efficient anchor for a surfactant molecule into the lipid interface. However as the hydrophilic nature of the surfactant is increased by more extensive ethoxylation the LAEs become less inhibitory than NPEs, inferring the NPEs to be more efficiently retained within the lipid interface. The mechanism behind this result may be due to the short, fluid alkyl chain on nonylphenol making the molecule easier to dissolve in the core of the lipid droplet, compared to the straight hydrocarbon chain of LAEs.

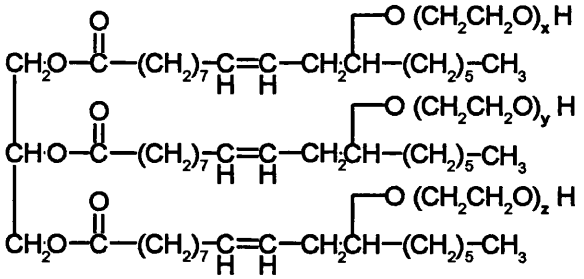
The same reasoning can also be related to the stronger inhibitory action of LAEs compared to NPEs with the same extent of ethoxylation below a HLB value of 13. The more efficient partitioning of the nonylphenol component in lipid could draw the surfactant further into the lipid droplet leaving less ethoxy groups at the interface relative to an LAE with comparable ethoxylation. The LAEs would then have a higher proportion of the ethoxy chain at the surface to mediate more effective inhibition of pancreatin via hydrophilic effects.

Insufficient surfactants were available to enable any conclusions to be drawn with regards to influence from the hydrocarbon chain of LAEs on inhibition of pancreatin activity. Varying the length of the alkyl chain by two carbons did change the potency

of inhibition exhibited by LAEs with the same ethoxylation number. However the difference remained minimal and related to the altered HLB value of the surfactant. The presence of a double bond in the alkyl chain did appear to increase inhibition, possibly by enhancing flexibility of the alkyl chain and thus partitioning into lipid.

4.8 Castor oil ethoxylated surfactants (including hydrogenated castor oil ethoxylates).

The ethoxylated forms of castor oil are widely used in pharmaceutical preparations as solubilisers, dispersants, emulsifiers and emollients. Two series of ethoxylated castor oil derivatives were obtained for testing, the Etocas series (castor oil ethoxylates) and the Croduret series (hydrogenated castor oil ethoxylates). Hydrogenation of the carbon to carbon double bond in castor oil forms the Croduret series, which have greater chemical stability compared to the castor oil ethoxylated surfactants. The general formula of a castor oil ethoxylated surfactant[180] is shown below:



The number of moles of ethylene oxide reacted per mole of castor oil is the sum of **x**, **y** and **z**, which in turn is expressed as the ethoxylation number.

The castor oil ethoxylates (Etocas and Croduret series) were assessed for potency of inhibition towards pancreatin activity by production of digestion profiles, as described in section 4.6. Results were examined in relation to the HLB value of the surfactants. The Croduret series, by comparison with Etocas surfactants, enabled examination for alteration in inhibitory activity due to hydrogenation of the double bond in castor oil. An attempt was also made to determine the HLB value of the surfactant Cremophor, also a hydrogenated castor oil ethoxylate, used extensively in Chapter 3.

4.8.1 Results and discussion

Figures 4.17 and 4.18 illustrate the digestion profiles produced using the Etocas and Croduret surfactants respectively, with the ethoxylation number and HLB value indicated for each surfactant. From comparison of the digestion profiles with the control, all castor oil ethoxylated surfactants markedly reduced pancreatin activity towards MCT (Figures 4.17-4.18).

Surfactants with a high degree of ethoxylation (HLB range 12.7 to 14.7) almost completely suppressed pancreatin activity throughout the hour assay (Figures 4.17-4.18). However if the assay had continued for a longer period, ranking of the surfactants in terms of potency of inhibition may have been possible from comparison of the duration of the lag phase. At HLB values above 14.7 inhibition started to decline within the assay period (i.e. Etocas-100 with a HLB of 16.5) (Figure 4.17). The surfactants with a low extent of ethoxylation, (Etocas-5 and Croduret-7) have HLB values of 3.9 and 5 respectively, and thus high lipophilic character. Both of these surfactants had a greater inhibitory effect on pancreatin activity than alcohol ethoxylates with the same number of ethoxy groups (comparison not shown). A possible explanation for their higher inhibitory effect may be due to strong attractive forces between the substantial castor oil component and MCT. Resultant adsorption of the hydrophobic castor oil moiety of the surfactant to the lipid interface could restrict binding of enzyme to the substrate, if surfactant concentration was sufficient to cover all of the interface.

Etocas-10 with a HLB value of 6.3 showed less inhibition (Figure 4.17) than 10-NPE / LAE surfactants (Figure 4.15c). The reduced inhibitory effect from the same number of ethoxy groups may be due to the manner of their distribution on the surfactant molecule. The castor oil molecule has three sites where ethoxylation is most likely to occur (on the hydroxyl groups), with a possibility of ethoxylation also at the site of the three carbonyl groups. In the case of alcohol ethoxylates all the ethoxy groups form a single chain upon one site. Therefore if inhibitory effect is due to a barrier formed by ethoxy groups at the lipid interface, the castor oil ethoxylates would require a higher degree of ethoxylation to build up the length of the ethoxy chains,

and thus reach the same inhibitory effect as an alcohol ethoxylate with a lower ethoxylation number.

Some evidence of this was shown upon comparison of the potency of inhibition of Etocas and Croduret surfactants with that of the NPEs, using the time taken for pancreatin to digest 2.5 % of MCT, $t_{2.5\,\%}$. Figure 4.19 illustrates the relationship between $t_{2.5\,\%}$ and HLB value for the castor oil derived surfactants, which can be seen to resemble the trend exhibited by the NPEs (also shown). Of those examined, the castor oil ethoxylate with the highest inhibitory effect had a HLB of approximately 14 and an ethoxylation number of 50; the most inhibitory NPE had an ethoxylation and HLB number of 15 (Figure 4.19). The surfactants have similar HLB values despite the wide difference in ethoxylation number, as the castor oil ethoxylates require extensive ethoxylation to offset influence from the high molecular weight lipophilic castor oil component of the surfactant.

The effect of hydrogenation of castor oil on inhibition of pancreatin activity was examined by comparison of Etocas and Croduret surfactants with identical ethoxylation numbers, 40 and 60. No difference in potency of inhibition was evident between digestion profiles of Etocas-40 and Croduret-40 whereas when 60 ethoxy groups were present, Etocas-60 was less inhibitory. These conclusions were drawn from examination of the profiles shown in Figures 4.17 and 4.18 using a larger scale. Etocas-60 and Croduret-60 both have a HLB value of 14.7 which appears to be near the HLB region where the potency of inhibition towards pancreatin starts to decline due to high hydrophilic character of the surfactant. The difference between profiles was small and hence may be due to experimental variation. Alternatively Croduret-60 may have retained potency of inhibition more efficiently than Etocas-60 due to increased molecular stability conferred by hydrogenation, however further data would be necessary to support this suggestion.

4.8.2 Examination of the potency of inhibition of Cremophor in relation to other castor oil derived ethoxylated surfactants.

Cremophor, a hydrogenated castor oil ethoxylate with an ethoxylation number of 45, was used in Chapter 3 to investigate the type of inhibition shown by non-ionic surfactants towards pancreatin activity. From subsequent work in this chapter the extent to which non-ionic surfactants inhibit pancreatin appears to be related to the HLB value of the surfactant. The HLB value of 14 to 16 stated for Cremophor in the product data sheet was in an area where Etocas and Croduret surfactants started to lose inhibition as hydrophilic character increased.

Personal communication with the manufacturer did not provide further information regarding a more precise HLB value for Cremophor. The potency of inhibition of the Etocas and Croduret surfactants appeared to be related to their HLB value. Using this relationship an attempt was made to determine a narrower HLB range for Cremophor and to assess the potency of inhibition of Cremophor in relation to other castor oil ethoxylated surfactants.

A digestion profile of MCT in the presence of Cremophor was produced under identical conditions to those used for the Etocas and Croduret series. The resulting profile was similar to the profiles from the Etocas and Croduret surfactants with ethoxylation numbers of 40 to 60. Enzyme activity in the presence of Cremophor was revealed to be almost completely suppressed throughout the hour assay, with total MCT digested not exceeding 3.5 % (data not shown).

The $t_{2.5\,\%}$ value from the digestion profile of Cremophor is represented in Figure 4.19 across a HLB range of 13.6 to 14.7. This HLB range was calculated for Cremophor using analytically determined saponification values stated in the product data sheet and Equation 4.1. The ricinoleic acid number[181] of 187.98 was used for the calculation as ricinoleic acid constitutes 90 % of the fatty acids in castor oil[182]. From compositional data of Cremophor a further HLB value of 13.6 was calculated via Equation 4.2 which fell within the same range given by Equation 4.1.

The trend between $t_{2.5\,\%}$ and HLB value for the Etocas / Croduret surfactants exhibits considerable scatter and could not be used with confidence to determine a HLB value for Cremophor. The position of $t_{2.5\,\%}$ from Cremophor (Figure 4.19) in relation

to $t_{2.5\,\%}$ from other castor oil derived surfactants suggests Cremophor to have a HLB value of approximately 14.5, which falls within the range specified by the data sheet and calculated via Equation 4.1. Figure 4.19 also reveals that Cremophor is not as inhibitory towards pancreatin as some of the other castor oil derived surfactants or NPEs examined.

4.9 Sorbitan esters and polyethoxylated sorbitan esters.

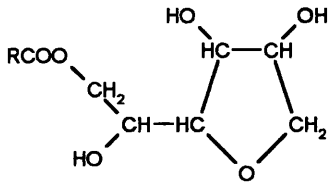
The sorbitan esters and the polyethoxylated sorbitan esters (PSEs) have become well established surfactants due to their low toxicity and wide range of applications in the pharmaceutical and food industry[165]. The sorbitan esters are effective water in oil emulsifiers and can act as wetting and dispersing agents for powders in lipophilic systems. When ethoxylated these surfactants display properties of oil in water emulsification and solubilization. The popularity of sorbitan esters and PSEs is due in part to the ease with which the two types can be combined to achieve the degree of emulsification required for a particular oil in water system.

Sorbitan esters are produced by the dehydration of sorbitol, to form a cyclic ether. The resulting anhydrous sorbitol has four alcohol groups remaining which are capable of undergoing esterification. Esterification can occur solely with fatty acids to form sorbitan esters or with fatty acids and ethylene oxide resulting in PSEs. The sorbitan esters and PSEs are described in terms of their degree of esterification with fatty acid, for example if only one alcohol group on the sorbitan moiety is esterified with fatty acid the surfactant is known as a monoester. The sorbitan esters and PSEs used in this study are monoesters or polyesters formed using one of the following fatty acids:

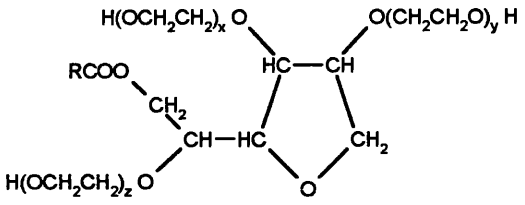
Common names	Structural Formula	Referred to in text as:
lauric acid	$CH_3(CH_2)_{10}COOH$	($C_{12:0}$)
palmitic acid	$CH_3(CH_2)_{14}COOH$	($C_{16:0}$)
stearic acid	$CH_3(CH_2)_{16}COOH$	($C_{18:0}$)
oleic acid	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$	($C_{18:1}$)

The general formula for a sorbitan monoester and a polyethoxylated sorbitan monoester are shown below as described by the supplier[183]:

Sorbitan Monoester



Polyethoxylated Sorbitan Monoester



where R = the alkyl group of the fatty acid.

$x + y + z$= the total number of moles of ethylene oxide (≡ to the ethoxylation number) reacted with one mole of sorbitan ester to form PSEs.

The ethoxy groups of a polyethoxylated sorbitan ester (PSE) will be distributed on the x, y and z positions shown in the diagram in the case of monoesters or all upon one position for triesters, although some literature sources[85, 165] suggest ethoxylation is also possible on the carbonyl groups of the fatty acids. Personal communication with Croda, the supplier of the majority of the sorbitan derived surfactants used in this study, confirmed ethoxylation was possible on the carbonyl group of the fatty acids for their products, although ethoxylation on the hydroxyl groups was favoured.

Sorbitan esters are classified as non-ionic surfactants, as although they are mainly dominated in character by the lipophilic fatty acid chains, a weak hydrophilic influence is conferred by the sorbitan moiety. The fatty acid chains give sorbitan esters high solubility in lipophilic systems which increases with the extent of fatty acid esterification. This is reflected in their HLB values which decrease from the monoesters down to the triesters.

The sorbitan esters (Crill and Span series) and the PSEs (Crillets and Tween series) were assessed for potency of inhibition towards pancreatin activity by production of digestion profiles, as described in section 4.6. Results were examined in relation to the ethoxylation number and HLB value of the surfactants (which ranged from 1.8 to 16.7); the extent of fatty acid esterification and the nature of the fatty acid component of the surfactant was also considered.

4.9.1 Results and discussion

Figure 4.20 illustrates digestion profiles produced in the presence of sorbitan monoesters and their counterparts with an ethoxylation number of 20 (20-PSEs). Sorbitan monoesters generally caused a slight depression in enzyme activity at steady state, with total MCT digested after one hour marginally decreased compared to the control (Figure 4.20).

The digestion profiles from the 20-PSEs exhibited significant suppression of pancreatin activity, shown as an extended lag phase; in addition less than 25 % of the total MCT available was digested within the one hour assay (Figure 4.20). Comparison of results in Figure 4.20 suggest ethoxylation is responsible for the extensive inhibitory activity of 20-PSEs towards pancreatin.

A trend between the length of the fatty acid chain and potency of inhibition was not apparent from profiles in Figure 4.20, however for both sorbitan monoesters and 20-PSEs the $C_{18:1}$ chain had a higher inhibitory effect than the $C_{18:0}$ chain. This is in agreement with results from the LAEs, where $C_{18:1}$ consistently showed increased inhibition compared to $C_{18:0}$ (Figure 4.15a, c & d).

Figure 4.21 illustrates digestion profiles from the complete series of PSEs tested. Inhibitory activity was noted to increase with the extent of ethoxylation of the PSEs, however the trend appeared random relative to the HLB values of the surfactants. A lag phase which lasted for the total duration of the assay occurred in the presence of $C_{18:0}$ and $C_{18:1}$ 20-PSEs (monoesters) (Figure 4.21) with a HLB value of approximately 15. The same potency of inhibition was shown by the $C_{18:0}$ and $C_{18:1}$ 20-PSEs (triesters) at a lower HLB value of 10 to 11 (Figure 4.21).

For a polyethoxylated sorbitan triester to exhibit the same HLB value as a polyethoxylated sorbitan monoester with an identical fatty acid chain, the triester requires a higher degree of ethoxylation to offset the lipophilicity conferred by the two additional fatty acid chains. To illustrate this point the $C_{18:1}$ 5-PSE (monoester) with only 5 ethoxy groups has a HLB of 10 whereas the $C_{18:1}$ triester with a HLB of 11 has 20 ethoxy groups. Digestion profiles produced in the presence of both surfactants are shown in Figure 4.21 where despite similar hydrophile / lipophile balance within the molecule, the triester is more inhibitory towards pancreatin.

Several reasons may be responsible for this effect. A triester may be a more efficient inhibitor than a monoester with the same HLB value due to the higher number of ethoxy groups available to form a more effective ethoxy barrier at the lipid interface. Alternately the additional fatty acids could enhance inhibition by promoting stronger hydrophobic binding between the surfactant and lipid interface, thus blocking access of pancreatin to substrate. Finally the larger size of the triester surfactant molecule may be involved by preventing enzyme / substrate binding via spatial effects.
Evidence for the existence of a lipophilic mechanism of inhibition is given by comparison of the potency of inhibition shown by the non-ethoxylated sorbitan triesters ($C_{18:0}$ and $C_{18:1}$) and their monoester counterparts (Figure 4.22). The increased inhibitory effect of the triesters is solely due to raised surfactant lipophilicity from the additional fatty acids. The pattern of inhibition from these lipophilic surfactants involves a decrease in both enzyme activity at steady state and total MCT digested at the end of the assay. The reduction in pancreatin activity does not show the characteristic lag phase seen with inhibition from ethoxylated surfactants (e.g. Figure 4.21, $C_{18:1}$ 5-PSE).
The conclusion from these comparisons is that the extent of inhibition of pancreatin activity may be related to the degree of ethoxylation and the lipophilic character of the surfactant. These two influences may act either in combination (as proposed in section 4.5) or independently depending on the structure of the surfactant. When assessing results from these sorbitan derived surfactants any influence on inhibition of pancreatin from the hydrophilic sorbitan ring was considered unlikely, other than through general spatial obstruction to enzyme binding.

4.10 Polyglycolized Glycerides.

Polyglycolized glycerides are ethoxylated esters made by an alcoholysis reaction of natural oils with polyethylene glycols. The polyglycolized glycerides have many pharmaceutical applications, however of particular interest to this study is their proposed ability to improve the bioavailability of hydrophobic drugs when administered orally[184]. The amphiphilic nature of the polyglycolized glycerides is suggested to enable formation of oil in water dispersions, miscible with cholesterol,

triglycerides and phospholipids present in the GI fluid. In addition a polyglycolized glyceride oil, Labrasol included in this study has been described as exhibiting good solvent capabilities and emulsification properties to aid formation of emulsions and microemulsions. The polyglycolized glycerides have the added advantage of solubility in vegetable oils and compatibility with gelatin capsule shells.

From the above description polyglycolized glycerides appear particularly suited for use in lipid-based formulations for hydrophobic drugs. Inhibition of pancreatin activity by these surfactants may therefore be especially relevant in terms of enhancement of the bioavailability of drugs via lipolysis. The polyglycolized glycerides (Labrafil series (C_{18} complex with PEG), Labrafac (C_8-C_{10} + PEG 400) and Labrasol (C_8-C_{10} + PEG 8)) were assessed for potency of inhibition towards pancreatin activity by production of digestion profiles, as described in section 4.6.

4.10.1 Results and discussion

Figure 4.23 illustrates digestion profiles produced in the presence of each polyglycolized glyceride. The medium chain polyglycolized glycerides ($C_{8:10}$) had little influence upon pancreatin activity, although a lag phase of short duration was seen with the 8-PEG version.

The long chain polyglycolized glycerides (6-PEG $C_{18:1}$ and $C_{18:2}$) depressed pancreatin activity at steady state with a decrease in total MCT digested upon completion of the assay compared to the control. The $C_{18:2}$ 8-PEG showed similar behaviour with the introduction of a short lag phase, probably due to higher hydrophilic character compared to the 6-PEG variants.

The inhibition of pancreatin activity by the $C_{18:1}$ and $C_{18:2}$ polyglycolized glycerides followed the same trend with HLB value as seen with the other surfactant groups already discussed. This may be due to the fact that the long chain polyglycolized glycerides are composed of natural oil in a complex with PEG. On the other hand the medium chain polyglycolized glycerides ($C_{8:10}$) showed little inhibition relative to their HLB numbers of 10 and 14, values which for other surfactant classes resulted in extensive enzyme inhibition. This may be a result of the composite nature of these products.

The polyglycolized glyceride ($C_{8:10}$) is present with free glycerides and probably free polyethylene glycol as well. As the surfactant is added to the assay by weight the mixture of constituents will have reduced the concentration of inhibitory substance present within the reaction system. Free polyethylene glycol would be expected to partition into the aqueous phase. More importantly the free medium chain glycerides could be assumed to be available at the lipid interface for hydrolysis by pancreatin and to enhance binding of colipase according to the colipase partitioning theory. This may explain the short lag phase seen, and a final extent of triglyceride digestion equivalent to that shown by the MCT control.

4.11 Influence of surfactant chemical structure upon inhibition of pancreatin.

The above investigation (section 4.7-4.10) has provided evidence to suggest that the chemical structure of the surfactant, in addition to the HLB value, was related to the potency of inhibition of a surfactant. The pattern of inhibition shown across a digestion profile had a particular character depending upon whether the lipophilic or hydrophilic moiety of the surfactant was dominant. This in turn suggested inhibition to be mediated by different mechanisms.

Potency of inhibition of NPEs was proposed to relate to partitioning of the surfactant molecule into the lipid interface and extensive inhibition was seen when the ethoxy chain was long enough to adopt a meander form. The resulting ethoxy mantle created around the lipid droplet could prevent enzyme binding by spatial and possibly detergent mediated effects.

The partitioning behaviour of the hydrophobic moiety of the alcohol ethoxylates into the lipid interface was proposed to be responsible for difference in inhibitory activity between NPEs and LAEs with similar ethoxylation numbers. The aromatic hydrophobe of NPEs was suggested to increase retention of surfactant within the lipid interface compared to the linear alkyl chain of LAEs.

The high potency of inhibition demonstrated by the castor oil ethoxylates was suggested to relate to the extensive hydrophobic castor oil component. In all cases digestion profiles from the castor oil ethoxylates displayed a lag phase indicating an initial delay in enzyme activity. Interestingly existence of a lag phase for lipophilic

members of a surfactant series was peculiar to the castor oil ethoxylates. All lipophilic members of the other surfactant groups tested did not exhibit a lag phase but suppressed enzyme activity across the whole digestion profile. Suppression of enzyme activity across the whole profile has been proposed to be a result of incorporation of surfactant molecules into the interface, where they effectively dilute the concentration of substrate available at the interface. The large size of the castor oil component and the close proximity of the ethoxylation sites to the fatty acid chains may prevent integration of these surfactants into the triglyceride interface. Instead a surfactant layer may be formed upon the MCT surface thus preventing initial enzyme / substrate binding resulting in a lag phase.

The number of sites of ethoxylation on a surfactant molecule may also be relevant to the extent of inhibition. If an identical number of ethoxy groups are distributed over several positions on a molecule as opposed to forming a single chain on one site reduced inhibition may be expected. This would be due to the longer single chain being able to protrude further from the lipophilic moiety of the surfactant, forming a more effective ethoxy mantle than several shorter chains. Further surfactants were required to test this hypothesis.

Comparison of sorbitan mono and triesters demonstrated that an increase in lipophilic character of the surfactant increased potency of inhibition. The same triester displayed a further enhancement in potency of inhibition when ethoxylated. This inferred that hydrophilic inhibition from ethoxylation and substrate dilution from the lipophilic moiety can occur at the same time to increase inhibition of pancreatin. This alone cannot however be taken as definite evidence of combined lipophilic and hydrophilic inhibitory mechanisms as results may simply be reflecting spatial obstruction from the larger surfactant structure to enzyme / substrate binding.

4.12 Conclusions from the investigation of inhibition of pancreatin activity by non-ionic surfactants.

From these investigations of various groups of non-ionic surfactants it is clear that under conditions of the standard pH-stat assay a relationship exists between the hydrophile-lipophile balance of a surfactant and the ability of the surfactant to inhibit

pancreatin activity towards MCT. At low HLB values surfactants have been proposed to inhibit pancreatin via lipophilic effects whereas when HLB is increased by ethoxylation hydrophilic inhibitory mechanisms may come into play. However the results suggest HLB not to be the only factor involved, as the structure of the hydrophobe also appears to have an influential effect on the potency of inhibition shown by a surfactant. In general for surfactants with the same extent of ethoxylation, inhibitory effect appears to increase with the size of the hydrophobic moiety.

It could be argued that the overall size of the surfactant molecule when present at the lipid-water interface may alone be sufficient to inhibit lipase purely by preventing access of colipase to the substrate. The altered nature of the digestion profiles according to HLB value of the surfactant however tends to suggest the situation is more complex. For surfactants with dominant hydrophilic character the effect upon digestion of MCT is to delay initial enzyme activity as indicated by a lag phase on the digestion profile. The lag phase is subsequently overcome after a variable period of time which tends to increase with the hydrophilic character of the surfactant.

Conversely the lipophilic surfactants from all groups, except the castor oil ethoxylates, decreased enzyme activity at steady state without evidence of a lag phase. Inhibition in this case was proposed not to be mediated by surface activity but to relate to a substrate dilution effect with surfactant molecules acting as inert spacers, reducing triglyceride concentration available at the interface for hydrolysis.

Lipophilic surfactants also tended to cause a reduction in the percentage of triglyceride digested upon completion of the assay compared to that shown by the MCT control. This may be a result of low HLB surfactants forming two phases of droplets, one of which is rich in surfactant. Any MCT incorporated in this phase could be expected to be held in a form unavailable to pancreatin, thus explaining the reduced level of triglyceride digestion observed at the end of the profile.

Throughout this study the mechanism of inhibition has been related to surfactants altering the availability of the substrate to the enzyme. A further point to remember is that MCT / surfactant emulsion systems could be expected to have different interfacial areas depending upon the HLB value of the surfactant, which could also influence pancreatin activity to some extent. The possibility of surfactant monomers in solution having a direct influence on the catalytic properties of lipase also cannot be ignored;

some detergent molecules have been observed in crystallographic studies[35] to locate at the entrance of the active site of lipase, bound to the hydrophobic part of the lid. In discussions throughout this chapter it has been assumed that digestion recorded related directly to pancreatin activity towards MCT. However as demonstrated by data in Chapter 5 certain surfactants are also available to digestion by pancreatin. Further experimentation would enable assessment of the extent to which this may have contributed to the results.

4.12.1 Relevance of findings to the use of surfactants within formulations.

This investigation has provided some guidance regarding selection of a surfactant for a lipid-based formulation in terms of minimizing possible inhibition of pancreatin activity:

- Surfactant series

For a surfactant series potency of inhibition can be predicted to some degree by testing a few surfactants spread across the HLB range. For example, results from the castor oil ethoxylates indicate that all members of the series have substantial inhibitory properties, irrespective of HLB value. This suggests the use of a castor oil based surfactant is best avoided.

- Minimum inhibitory HLB value within a surfactant series

Through a homologous series of surfactants, as the degree of ethoxylation is increased a point is reached where the hydrophilic and lipophilic moieties of the surfactant balance for a particular emulsion system. The degree of hydration of the interfacial film at this balance point brings the curvature of the adsorbed monolayer to near zero, the oil-water interfacial tension is at a minimum and the resulting oil in water emulsion exhibits maximum stability[170].

From investigation of the NPEs, Figure 4.4 shows inhibition of pancreatin activity to be at its lowest at a HLB value of approximately 11, which corresponds to the surfactant, 6-NPE. This surfactant may possess low lipophilic influence, in combination with an ethoxy chain too short to mediate inhibitory activity. Another

possibility is that 6-NPE may be the member of the surfactant series where the hydrophilic and lipophilic moieties of the surfactant are most evenly balanced at the interface under conditions of the standard pH-stat assay. The emulsion thus formed with 6-NPE, from the discussion above, would be expected to have a low interfacial tension. Lipase has already been shown to be prevented from binding to lipid due to high interfacial pressure and colipase was proposed to overcome this inhibition by increasing the interfacial pressure at which lipase could bind[176]. A surfactant capable of reducing interfacial tension could thus be assumed to promote lipase binding, hence inhibitory effects from the surfactant would be reduced.

If the same behaviour was shown through a series of surfactants suitable for use in a lipid-based formulation, the surfactant showing the lowest potency of inhibition could be chosen. A possible disadvantage of this approach is the need to assess several members of a particular surfactant series, across a wide range of HLB values, to enable detection of the HLB value where surfactant is most evenly balanced at the interface and thus least inhibitory.

- Length of the ethoxy chain

Results from the alcohol ethoxylated surfactants have shown that when the hydrophilic moiety of the surfactant was dominant, the potency of inhibition of the surfactant rapidly increased with each additional ethoxy group (Figure 4.6). Use of a surfactant with the shortest ethoxy chain possible, (and the properties required for the lipid-based formulation), would appear prudent in terms of minimization of interference with pancreatin activity.

These guidelines may lead to selection of the surfactant least likely to impede enzyme activity however the surfactant chosen on this basis may not provide the emulsification properties required by the lipid-based formulation. An interesting field to investigate would be the inhibitory capability of surfactant combinations such as mixtures of sorbitan esters with the polyethoxylated variants, which when combined enable selection of any HLB value.

4.13 Physicochemical characterization of surfactants.

The non-ionic surfactants utilized in this chapter were commercial surfactants known to be of variable composition. During the manufacturing process the addition of ethylene oxide to the hydrophobic moiety of the surfactant usually results in a Poisson distribution of the length of the ethoxy chains[185]. The properties of the surfactant material produced will therefore be dependent on the average extent of ethoxylation. HLB values used in this investigation for correlation with potency of surfactant inhibitory behaviour were based upon the ethoxylation number stated by the manufacturer. This number should be representative of the mean number of ethoxy groups on the surfactant molecule. To validate the HLB values used to compare the behaviour of surfactants, the mean degree of ethoxylation was determined for the majority of the alcohol ethoxylated surfactants included in the study. This was achieved using the techniques of cloud point determination and ^{1}H NMR.

4.13.1 Determination of cloud point temperature.

When an ethoxylated non-ionic surfactant is dissolved in water, solubility of the surfactant relies upon interactions between the hydrophilic ethoxy chain and water. If the temperature of the solution is gradually increased hydration forces between the ethoxy chain and water weaken, with a consequent reduction in solubility[171]. Eventually a temperature is reached at which the hydrophilic forces are no longer sufficient to maintain solubility of the surfactant in water and cloudiness is seen due to separation of the solution into two phases. The term cloud point[185] is used to describe the temperature at which this cloudiness occurs.

The cloud point temperature closely reflects the extent of ethoxylation of a surfactant and for materials with the same hydrophobe correlation between cloud point and ethoxy group content is quite precise[185]. Due to this statement of precision between cloud point and the degree of surfactant ethoxylation, cloud point determination was attempted for selected NPEs (Dowfax, Arkopal and Lutensol AP series) and LAEs (Lutensol AO and AT series) used in the inhibition study.

Method

For each surfactant a 1 % $^{w}/_{v}$ solution was prepared using distilled water at 4°C. After thorough mixing a description was taken of the resulting surfactant and water mixture. Stirring was maintained as the integral heating element of the magnetic stirrer gradually increased the temperature of the solution. Temperature was indicated by a thermometer suspended within the solution.

Temperature was recorded when slight clouding was first observed and again upon definite clouding which tended to occur suddenly. To aid determination of clouding a black background was used behind the solution.

Results

The temperature of definite clouding was taken to represent cloud point temperature under the experimental conditions used. The temperature when clouding was first observed allowed ranking of surfactants which exhibited identical cloud point temperatures.

According to the above definitions the NPEs and LAEs tested were listed in ascending order of cloud point temperature (Table 4.3). The results enabled ranking of surfactants with manufacturer quoted HLB values of 12 to 15 inclusively, in terms of degree of ethoxylation. It was not possible to determine cloud points for surfactants stated to have chain lengths of between 2 and 7 ethoxy groups as these surfactants were not soluble in an aqueous system. However using the principle that surfactants with less ethoxy groups display characteristics of higher lipophilicity, the description of the surfactant solution at 4°C (Table 4.3) served to rank the surfactants. Surfactants ranked in this manner showed increased dispersibility in an aqueous system as the ethoxylation number specified by the manufacturer increased. The solutions of highly hydrophilic NPEs with an ethoxylation number stated by the manufacturer of 20 and above remained clear upon boiling. This result gave support to the stated length of the ethoxy chains, as clouding is known not to occur below the boiling point of water when ethylene oxide constitutes > 75 % of the surfactant molecule[185]. The surfactants tested must therefore have a minimum of 15 ethoxy groups as with any less the ethoxy chain would not exceed 75 % of the total composition of a nonylphenol ethoxylated surfactant molecule.

Overall the cloud point temperatures supported the ethoxylation number stated by the manufacturer. The only exceptions were 7.5-NPE which the experimental cloud point indicated to be closer to an average of 8 ethoxy groups and 15-NPE, the cloud point of which suggested a lower extent of ethoxylation.

The cloud point determinations have thus given increased confidence in the HLB values calculated from the ethoxylation numbers stated by the manufacturer and hence in the correlation between potency of inhibition of the surfactant and HLB value.

Surfactant (series + ethoxylation number from manufacturer)	HLB value	Ethoxy groups (^{1}H NMR)	Cloud point (°C)	Solution appearance at 4°C
2-NPE (Dowfax)	5.7	2.0		Large oily droplets
4-NPE (Dowfax)	8.9	4.0		Coarse dispersion
4-NPE (Arkopal)	8.9	3.5		Fine, white dispersion
5-NPE (Dowfax)	10.0	5.0		Fine, white dispersion
6-NPE (Dowfax)	10.9	6.0		Fine, white dispersion
6-NPE (Arkopal)	10.9	5.5		Hazy, white suspension
7-NPE (Dowfax)	11.7	7.0		Hazy, white suspension
8-NPE (Arkopal)	12.3	8.0	16	Clear solution
7.5-NPE (Dowfax)	12.0	8.0	17	Clear solution
10-NPE (Dowfax)	13.3	9.5	55	Clear solution
10-NPE (Arkopal)	13.3	10.0	63	Clear solution
11-NPE (Arkopal)	13.8	11.0	70	Clear solution
12-NPE (Dowfax)	14.1	11.5	77	Clear solution
15-NPE (Arkopal)	15.0	13.5	92	Clear solution
14-NPE (Lutensol)	14.7	14.0	96	Clear solution
20-NPE (Lutensol)	16.0	19.0	>99	Clear solution
23-NPE (Arkopal)	16.4	21.0	>99	Clear solution
30-NPE (Arkopal)	17.1	29.5	>99	Clear solution
3-LAE (Lutensol AO)	8.0	3.0 C_{13}		Immiscible with water
8-LAE (Lutensol AO)	12.5	8.0 C_{14}	55	Clear solution
10-LAE (Lutensol AO)	13.5	10.0 C_{14}	78	Clear solution
11-LAE (Lutensol AT)	13.0	11.0 C_{17}	87	Clear solution, clouding first observed at 65°C
12-LAE (Lutensol AO)	14.3	12.0 C_{14}	87	Clear solution, clouding first observed at 79°C

Table 4.3 Characterization of the degree of ethoxylation of alcohol ethoxylated surfactants via cloud point temperatures and ^{1}H NMR spectroscopy.

4.13.2 ^{1}H NMR analysis

^{1}H NMR (NMR) provides a method for identification of the number of protons present within a surfactant molecule in terms of their chemical environment, thus enabling prediction or confirmation of surfactant structure. NMR is commonly employed for studies of alcohol ethoxylated surfactants to determine the ratio of alkyl to ethoxy protons[185]. NMR spectrometry was thus applied to the alcohol ethoxylates for which cloud point determinations had been attempted, as an additional method to investigate the length of the surfactant ethoxy chain.

Method

A NMR spectrum was produced for each surfactant listed in Table 4.3 using one of the spectrometers listed below:

Jeol JNM EX400 Fourier Transform Nuclear Magnetic Resonance Spectrometer at an observation frequency of 399.78 MHz.

Jeol JNM GX270 Fourier Transform Nuclear Magnetic Resonance Spectrometer at an observation frequency of 270.16 MHz.

For each surfactant tested approximately 20 mg of sample was dissolved in 3 ml of $CDCl_3$ and checked to ensure no insoluble impurities were present. The surfactant sample solution was then introduced into precision ground tubes of 5 mm internal diameter to a depth of approximately 2 cm. NMR spectra were taken at a temperature of 22°C using tetramethylsilane as an internal reference compound.

Interpretation of spectra

Tables[186] specifying expected chemical shift values for ^{1}H protons within a specific chemical environment were used to assign the signals to molecular structure; the ^{1}H chemical shift values used are included in Table 4.4, expressed in parts per million (ppm).

Structure	δ_H
CH_3-R	0.9
R-CH_2-R	1.4
CH-R	1.5
R-CH_2-Ar	2.7
R-CH_2-OR	3.4
R-CH_2-OH	3.6
R-CH_2-O-Ar	4.3
R-OH	0.5-4.5
Ar-OH	4.5-10
suspended HDO	~4.7
Aromatic H	6-9

Table 4.4 ^{1}H NMR chemical shifts[186] for proton indicated in bold.
R = alkyl group, Ar = aryl group. Values will normally be within 0.2 ppm unless electronic or anisotropic effects from other groups are strong[186].

The nonylphenol ethoxylated surfactants.

A typical ^{1}H NMR spectrum of a nonylphenol ethoxylated surfactant (obtained from a 10-NPE sample) in $CDCl_3$ is shown in Figure 4.24 with assignments from chemical shift values in Table 4.4 indicated. Upon the spectra from all NPEs tested signals were exhibited in three regions, 7.2-6.8 ppm, 4.1-3.6 ppm and 1.6-0.5 ppm. The aromatic protons resonate in the 7 ppm region (peaks i and h) whilst the signals at 4.1-3.6 ppm (peaks e, f, and g) were proposed to be due to the ethoxy protons. The signals between the chemical shift values of 1.6 and 0.5 ppm (peaks a, b, and c) were assigned to the alkyl protons.

A NMR spectrum of nonylphenol was used to further verify that the resonance seen at 1.6 to 0.5 ppm related to the alkyl protons. Nonylphenol produced signals only within the alkyl region (1.6 to 0.5 ppm) and the aromatic region (7.2-6.8 ppm) confirming the position of resonance predicted for ethoxy protons (4.1-3.6 ppm) upon the spectra of the NPEs. Nonylphenol exhibited the same resonance pattern as NPEs in the region of 1.6 to 0.5 ppm although the intensities of the peaks were altered, suggesting nonylphenol to have a different branched form of the nine carbon alkyl chain compared to the NPEs.

Ethoxy group protons

The ethoxy protons have already been assigned to signals in the 4.1 to 3.6 ppm region. In each spectrum a major peak was evident at 3.6 ppm (peak e) which

increased in intensity when spectra from surfactants with longer ethoxy chains were compared. As this peak represented the extent of surfactant ethoxylation a means of calibrating the corresponding integral trace was required.

Calibration was achieved by use of two multiplets (peaks f and g) shifted downfield (at approximately 4.1 and 3.85 ppm) to the major ethoxy peak. These multiplets were due to the protons from the ethoxy group adjacent to the aromatic ring. The signals from these protons were separated from the major ethoxy peak due to a deshielding effect from the aromatic ring known as π bond anisotropy[187].

Determination of the mean degree of ethoxylation of each nonylphenol ethoxylated surfactant was thus approached by measurement of the integral trace step for peak f known to correspond to two protons. Division of the millimeter value for one proton into the variable peak e trace gave the number of ethoxy protons present. The four ethoxy protons represented by peaks f and g were added to this figure and the total divided by four to determine the complete number of ethoxy groups present on the surfactant.

The calibration procedure was repeated for each spectrum as the integral trace is spectrum specific. The results were expressed to the nearest half ethoxy group and are shown in Table 4.3.

Alkyl protons

An attempt was made to confirm the identity of the alkyl chain using the NMR spectra. The number of alkyl protons present was determined from the three step integral trace above peaks a, b, and c (1.6 and 0.5 ppm) using the same calibration measurement employed to predict the number of ethoxy groups from peak e. For all NPEs examined the signal at 1.6 to 0.5 ppm confirmed the existence of 19 protons (error ± 1 proton) which corresponded to a nine carbon chain.

From chemical shift values (Table 4.4) the assumption can be made that peak a relates to methyl protons, peak b to methylene protons and peak c to methine protons. From comparison of the relative intensities of these peaks all spectra appear to exhibit an approximate proton ratio of $9(CH_3):8(CH_2):2(CH)$. The high proportion of methyl protons therefore suggests branching of the alkyl chain.

Commercial nonylphenol surfactants are described in the literature as typically containing a branched form of the alkyl chain. The chain does not tend to differ in

terms of molecular weight but as it is usually formed from a propylene trimer a mixture of isomers is possible[185]. The NMR results suggest all NPEs tested in this study have a branched chain structure, a possible arrangement for which is shown in Figure 4.24.

The hydroxyl proton

The chemical shift value of the hydroxyl proton attached to the terminal ethoxy group can vary between 0.5 and 4.5 ppm and may be represented on the spectrum in a broadened form, depending on sample concentration and hydrogen bonding[186]. To confirm the resonance position of the hydroxyl proton several spectra from NPEs and LAEs were repeated after shaking the sample with D_2O. This is expected to result in the exchange of the hydroxyl protons present for deuterium causing disappearance of the OH signal.

In each case the result of the deuterium shake was to remove the broad bump seen between 2 to 3 ppm on spectra from NPEs (e.g. Figure 4.24, peak d) and LAEs. A further signal was seen to occur at 4.8 ppm corresponding to HDO. This test therefore confirmed the position of the hydroxyl proton, allowing subsequent adjustment of the predicted number of alkyl protons if the integral also included the resonance area of the hydroxyl proton.

<u>The linear alcohol ethoxylates</u>

A typical ^1H NMR spectrum of a linear alcohol ethoxylated surfactant (obtained from an 11-LAE sample) in $CDCl_3$ is shown in Figure 4.25 with assignments from chemical shift values in Table 4.4 indicated. The spectra from the LAEs exhibited signals within two regions, 3.3 to 3.8 ppm and 1.6 to 0.8 ppm. The ethoxy protons were all proposed to be represented by peak f (3.7 ppm) whilst the alkyl protons were represented by peaks a, b and c in the region of 1.6 to 0.8 ppm.

The triplet signal at 3.4 ppm (peak e) was due to the terminal methylene before the start of the ethoxy chain[187]. As this peak was known to represent 2 protons the measurement in millimeters of the corresponding step of the integral trace was used to determine the number of protons in the ethoxy and alkyl regions. If the integral trace above peak e was not well defined upon a particular spectrum the integral trace above the preterminal methylene at 1.6 ppm (peak c) provided an alternative means of

calibration. Each spectrum was produced in duplicate as sharp integrals were not always achieved.

The number of ethoxy groups predicted via the NMR data for each LAE tested corresponded to the value quoted by the manufacturer and results are shown in Table 4.3. The alkyl chain of the LAEs tested was quoted by the manufacturer as being comprised of between 13 to 15 carbons for the Lutensol AO series and 16 to 18 for the Lutensol AT series. The NMR spectra indicated the mean length of the alkyl chain (Table 4.3), which for each LAE tested fell within the range specified by the manufacturer. In terms of the structure of the alkyl chain the spectra were not able to confirm this due to distortion of the integral above the methyl signal. However LAEs are made from alcohol starting material which is normally linear[185] and the integral step ratios on the NMR spectra did not suggest a branched structure.

Conclusions from NMR spectroscopy.

In general the NMR spectra indicated the mean length of the ethoxy chain to be identical to the ethoxylation number stated by the manufacturer. However for nine NPEs tested NMR data did not agree with the values quoted by the manufacturer, although the results only differed between 0.5 to 2 ethoxy groups. For all but one of these surfactants the number of ethoxy groups predicted by NMR was lower than the manufacturer value. This is not surprising given the fact that NMR determines the mean length of the ethoxy chain whereas the ethoxylation number represents the number of moles of ethylene oxide reacted with one mole of the alcohol.

The NMR spectra were able to confirm the mean number of carbons in the alkyl chain of the alcohol ethoxylates. In addition NPEs appeared to have a branched alkyl chain structure whereas spectra from LAEs suggested a linear chain.

4.14 Relationship between results from surfactant characterization and potency of inhibition shown by the NPEs.

The proposed relationship between surfactant HLB value and inhibitory behaviour of alcohol ethoxylated surfactants towards pancreatin had been based upon the ethoxylation number stated by the manufacturer, which was assumed to be equivalent to the mean length of the ethoxy chain. As the NMR data had revealed certain surfactants to have a different number of ethoxy groups compared to the ethoxylation number quoted by the manufacturer, Equation 4.2 was used to calculate a HLB value which reflected the length of the ethoxy chain determined by NMR.

To examine whether the correlation between potency of inhibition and HLB value had been affected by these discrepancies in the assumed degree of ethoxylation, use was made of the parameter $t_{\%}$. The time taken to reach 5 % MCT digestion ($t_{5\,\%}$) in the presence of NPEs was represented as a function of surfactant HLB value determined via the ethoxylation number quoted by the manufacturer (Figure 4.26a) and from the mean length of the ethoxy chain from the NMR data (Figure 4.26b).

From examination of Figure 4.26 a and b it was evident that the relationship between potency of inhibition and surfactant HLB value from the NMR data (Figure 4.26b) exhibited less scatter than when HLB values calculated from data supplied by the manufacturer were used (Figure 4.26a). This result gave further confirmation of the proposed correlation between surfactant HLB value and inhibitory behaviour of NPEs towards pancreatin. In addition the correlation gave further weight to the NMR predictions which also coincided with ethoxy ranking via the cloud point temperature method.

Figure 4.1 Comparison of pancreatin activity towards MCT (450 mg) in the presence of 10 % $^{w}/_{w}$ (with respect to MCT) NPEs with ethoxylation numbers from 2 to 12.

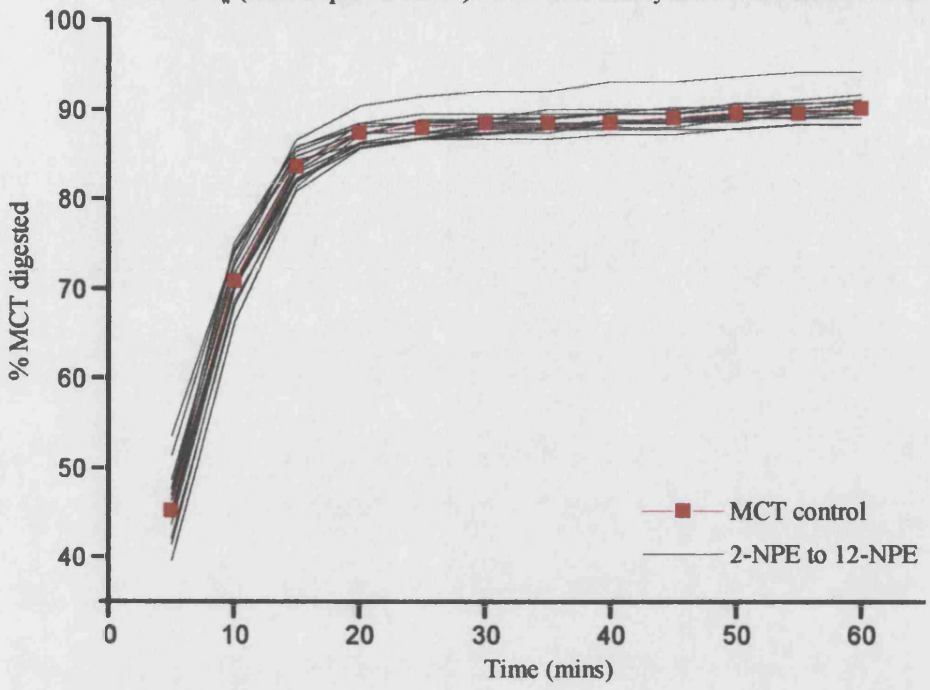


Figure 4.2 Comparison of pancreatin activity towards MCT (400 mg) in the presence of 20 % $^{w}/_{w}$ (with respect to MCT) NPEs with ethoxylation numbers from 2 to 12.

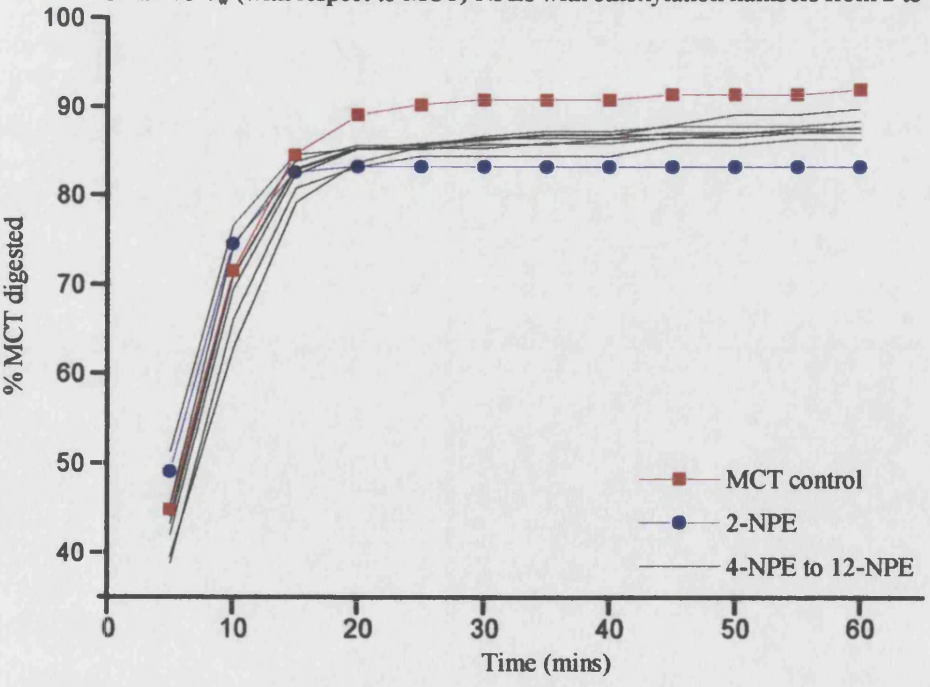


Figure 4.3a Comparison of pancreatin activity towards MCT (1 g) in the presence of 50 % $^{w}/_{w}$ (with respect to MCT) NPEs (1 g) with ethoxylation numbers from 0 to 6.

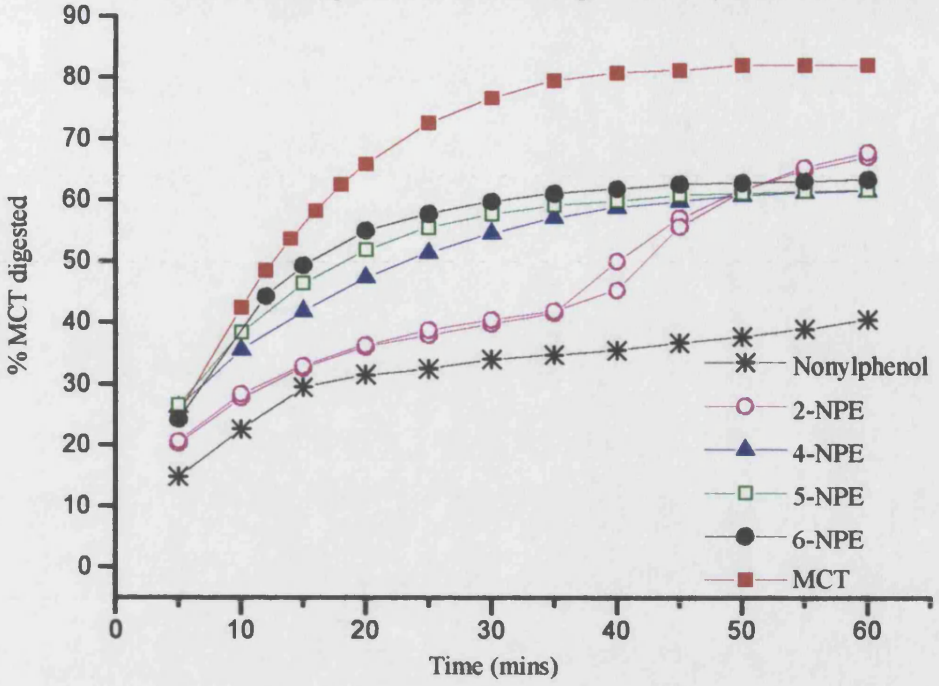


Figure 4.3b Comparison of pancreatin activity towards MCT (1 g) in the presence of 50 % $^{w}/_{w}$ (with respect to MCT) NPEs (1 g) with ethoxylation numbers from 6 to 12.

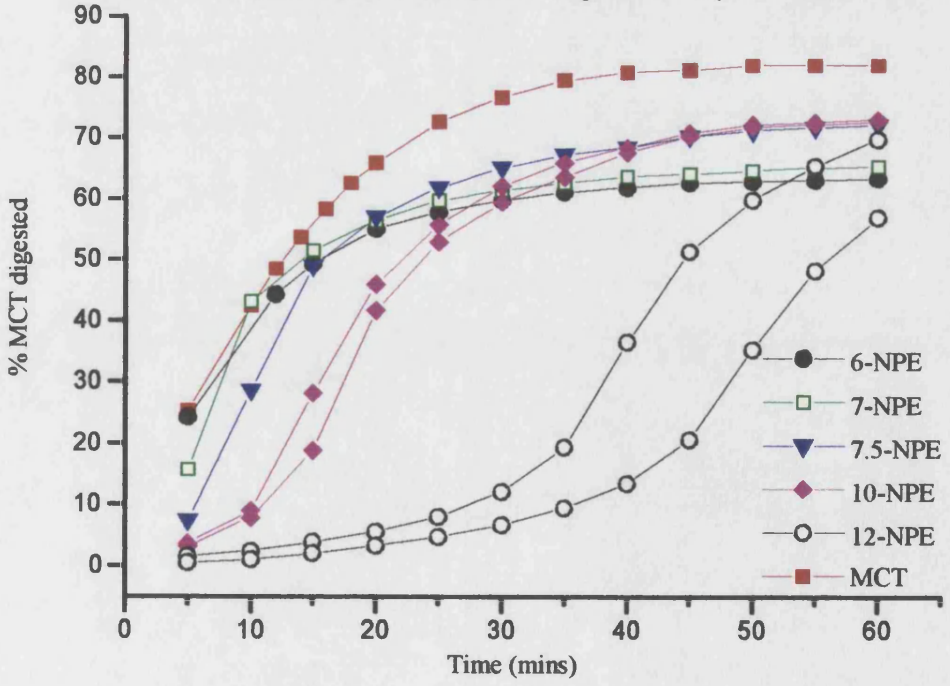


Figure 4.4 $t_{10\,\%}$ and $t_{25\,\%}$ as a function of surfactant HLB value for NPEs examined in Stage A of the investigation.

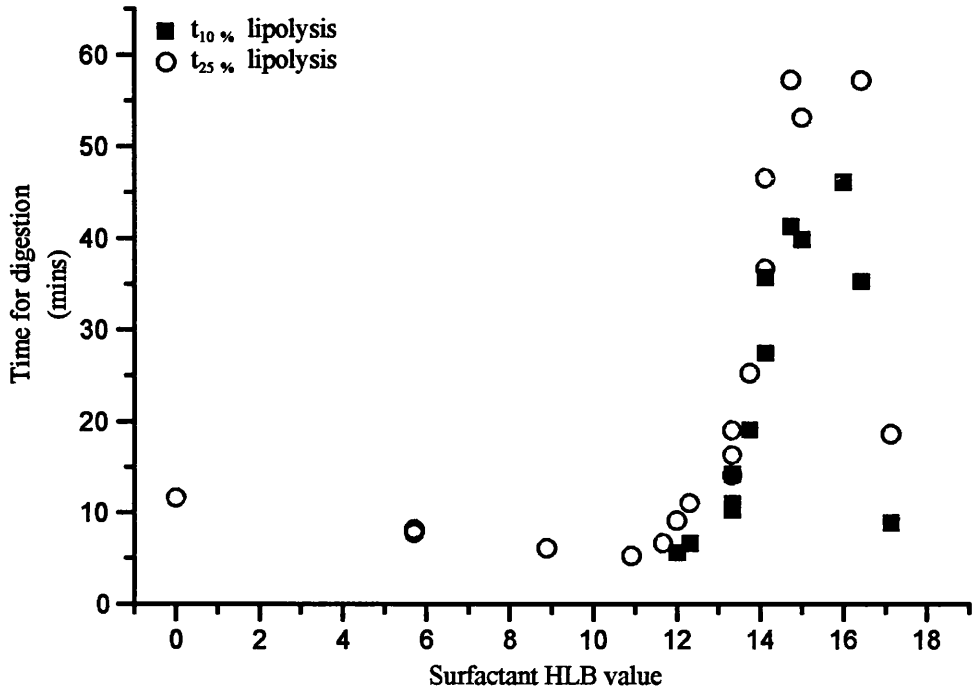


Figure 4.5 $t_{2.5\,\%}$ and $t_{5\,\%}$ as a function of surfactant HLB value for NPEs examined in Stage B of the investigation.

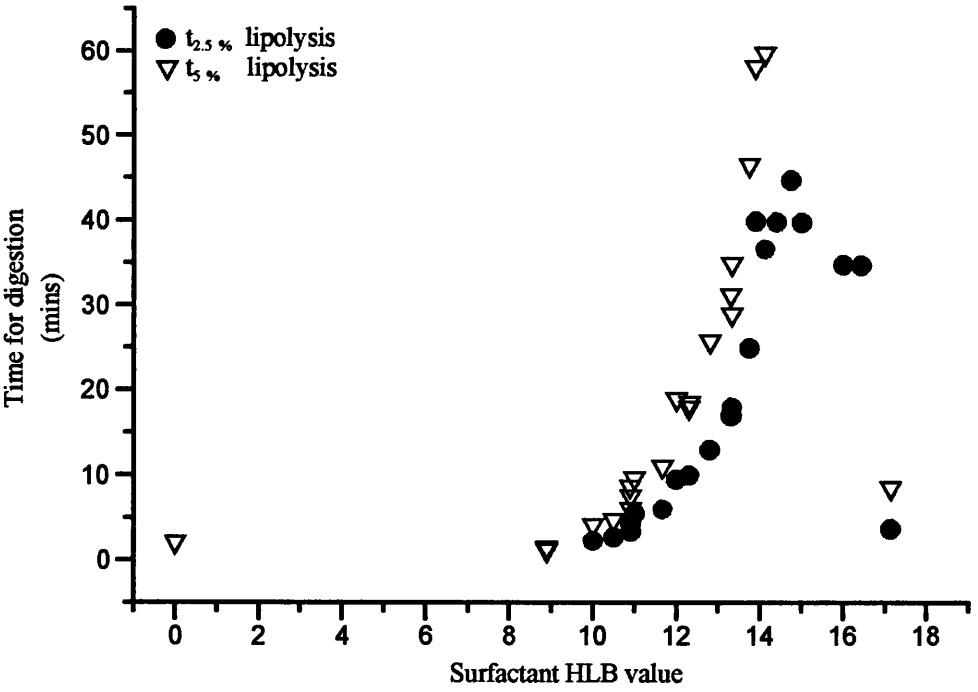


Figure 4.6 The exponential relationship between $t_{2.5\,\%}$ and surfactant HLB values from 10 to 15 for NPEs examined in Stage B of the investigation.

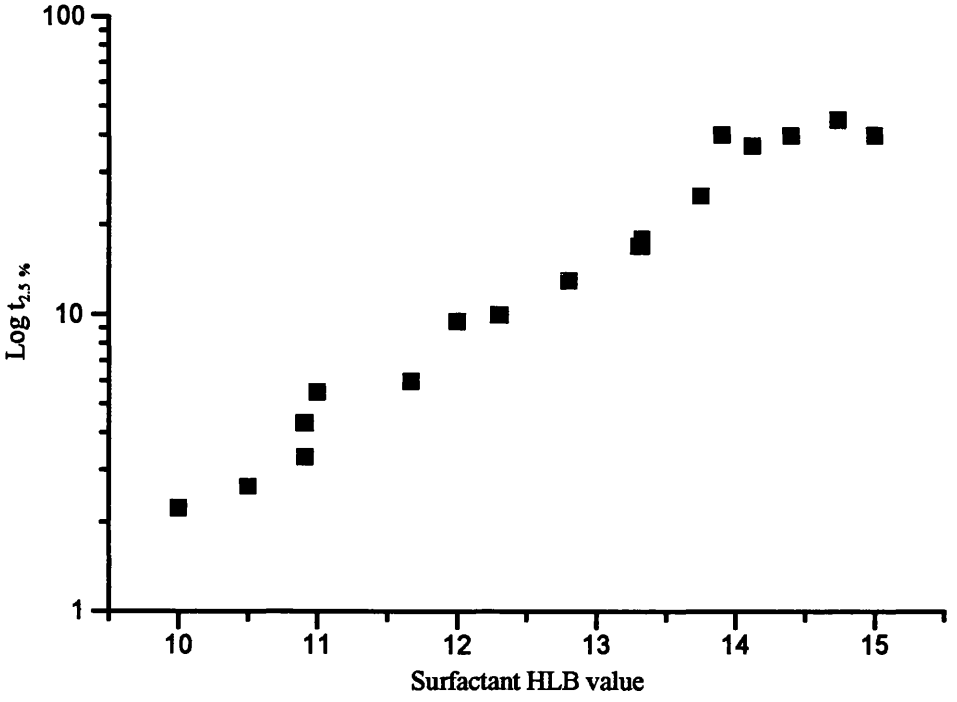


Figure 4.7 Comparison of $t_{25\,\%}$ as a function of surfactant HLB value for NPEs examined in Stage A and B of the investigation.

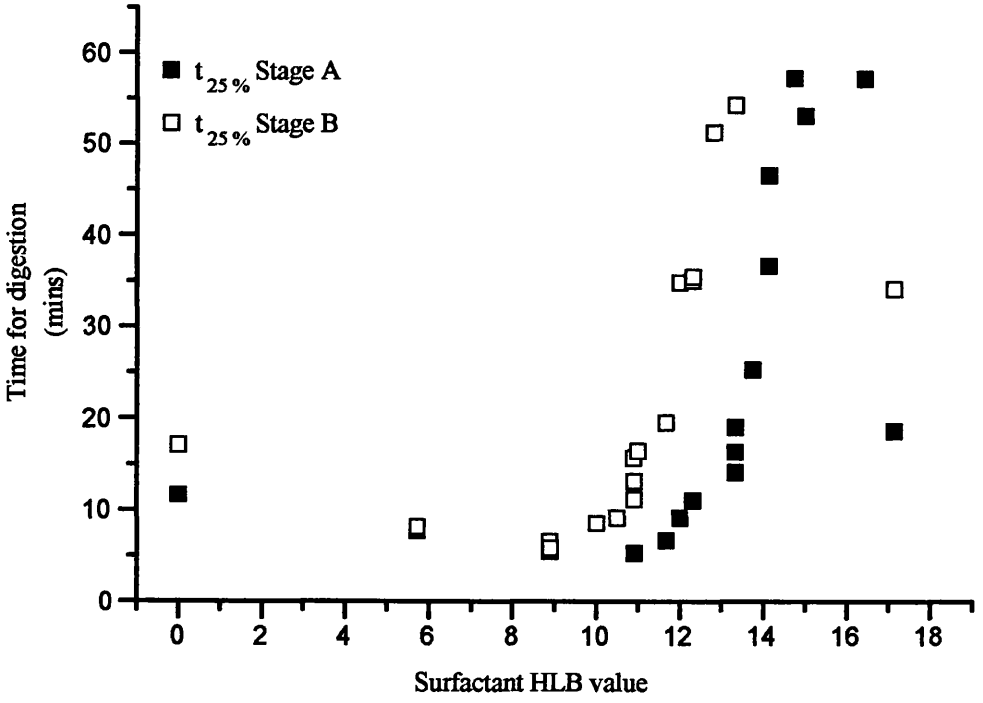


Figure 4.8 Digestion profiles of MCT (1 g) illustrating the activity of different batches of pancreatin towards substrate in the absence of NPEs.

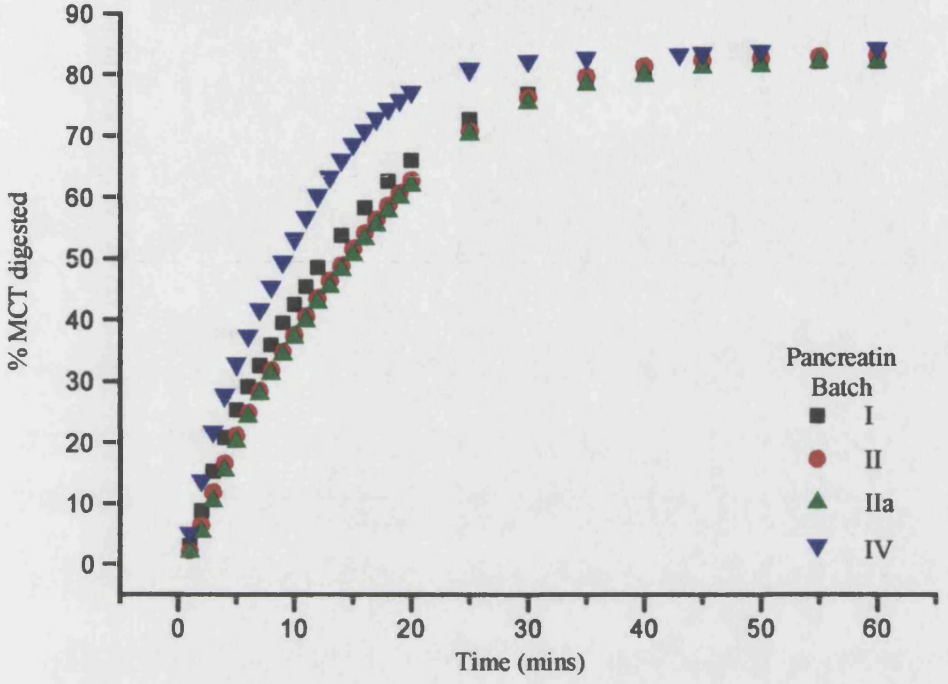


Figure 4.9 Digestion profiles of MCT (1 g) in the presence of 10-NPE illustrating the susceptibility of different batches of pancreatin to inhibition by the surfactant.

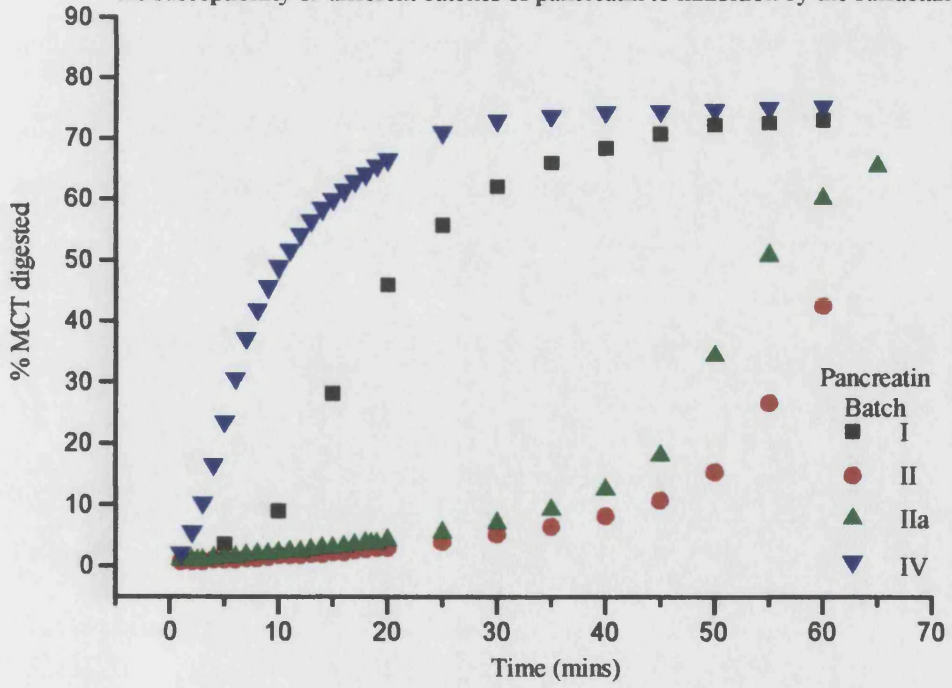


Figure 4.10 Digestion profiles of MCT (1 g) in the presence of 12-NPE illustrating the susceptibility of different batches of pancreatin to inhibition by the surfactant.

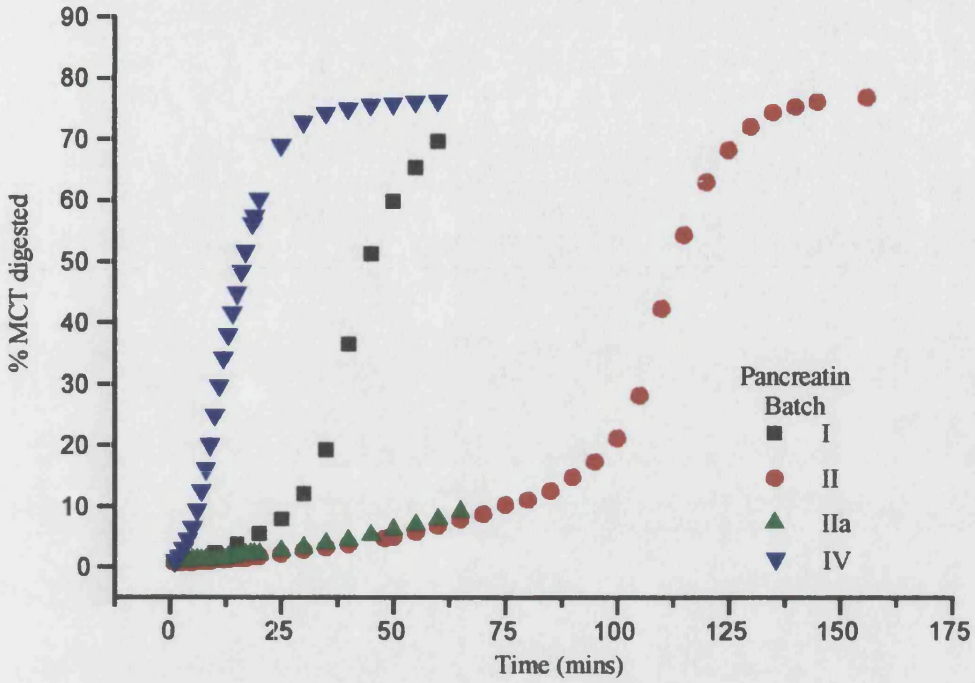


Figure 4.11 Duration of the lag phase as a function of surfactant HLB value when using different batches of pancreatin to digest identical MCT / NPE mixtures.

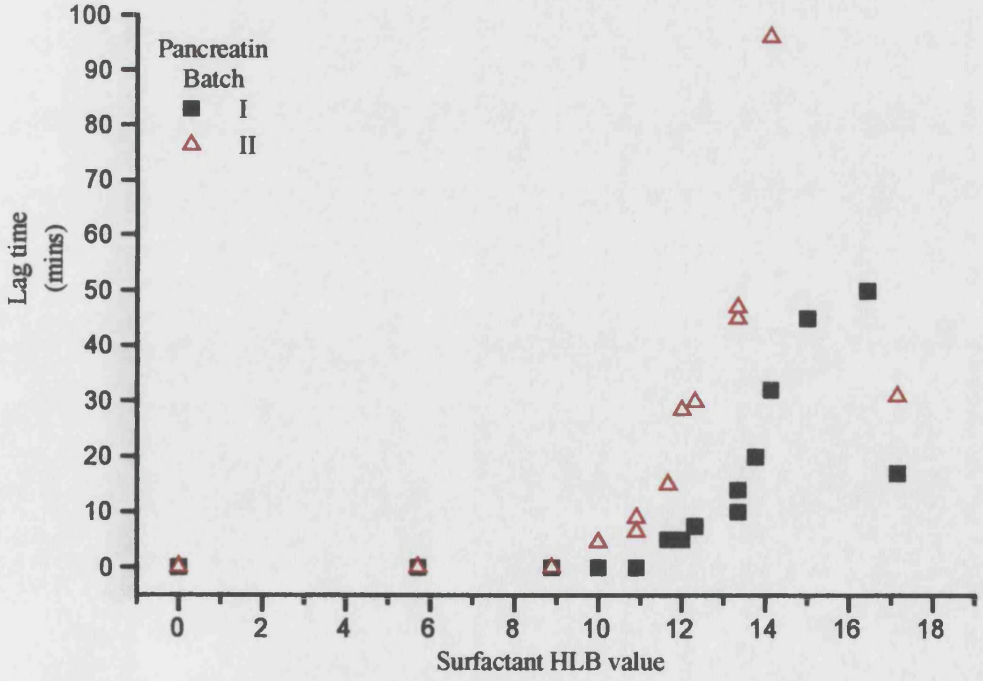


Figure 4.13 Comparison of pancreatin activity towards MCT (1 g) in the presence of LAEs (1 g) (Brij Series).

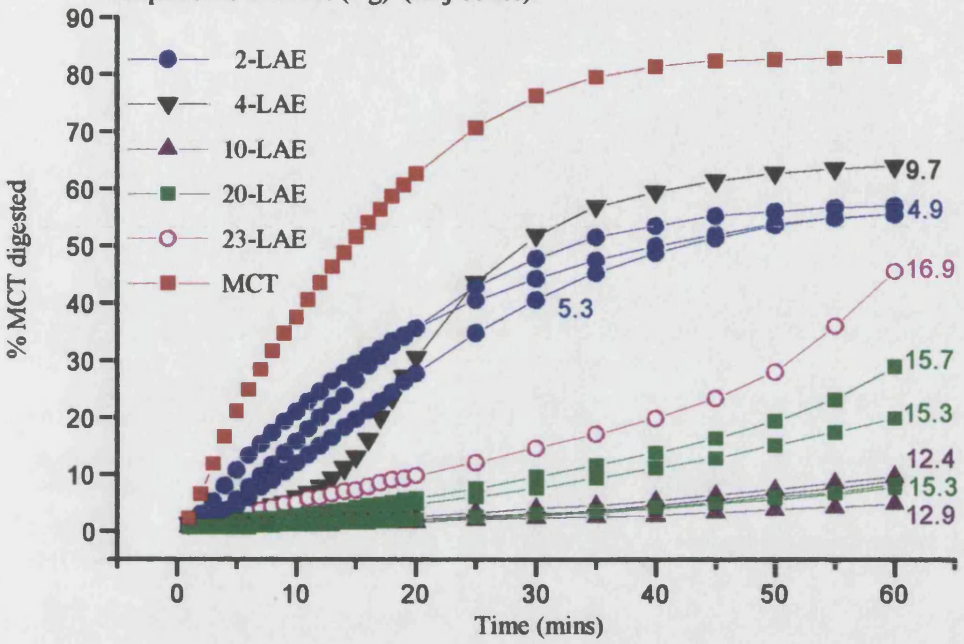
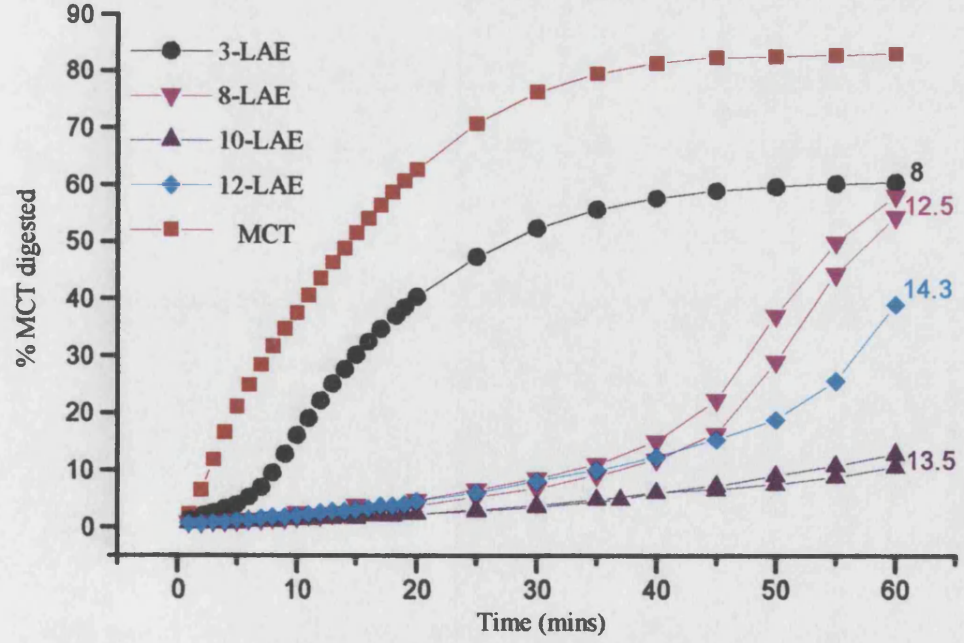


Figure 4.14 Comparison of pancreatin activity towards MCT (1 g) in the presence of LAEs (1 g) (Lutensol AO Series).



NB - HLB values of the surfactants are indicated at the end of the profiles.

Figure 4.15a Comparison of pancreatin activity towards MCT (1 g) in the presence of alcohol ethoxylated surfactants (1 g) with an ethoxylation number of 2.

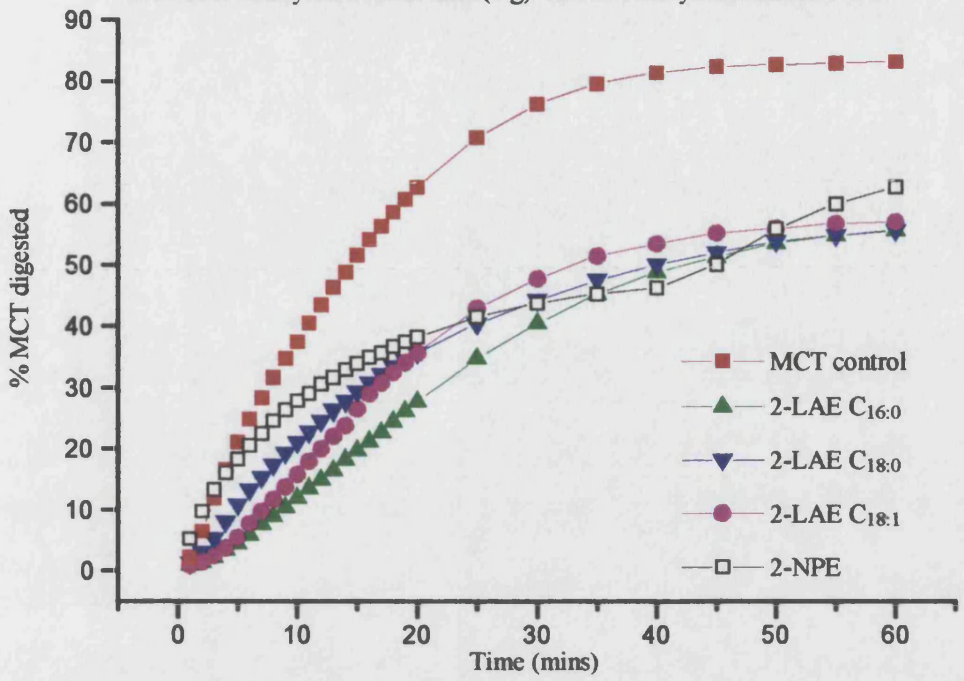


Figure 4.15b Comparison of pancreatin activity towards MCT (1 g) in the presence of alcohol ethoxylated surfactants (1 g) with an ethoxylation number of 4.

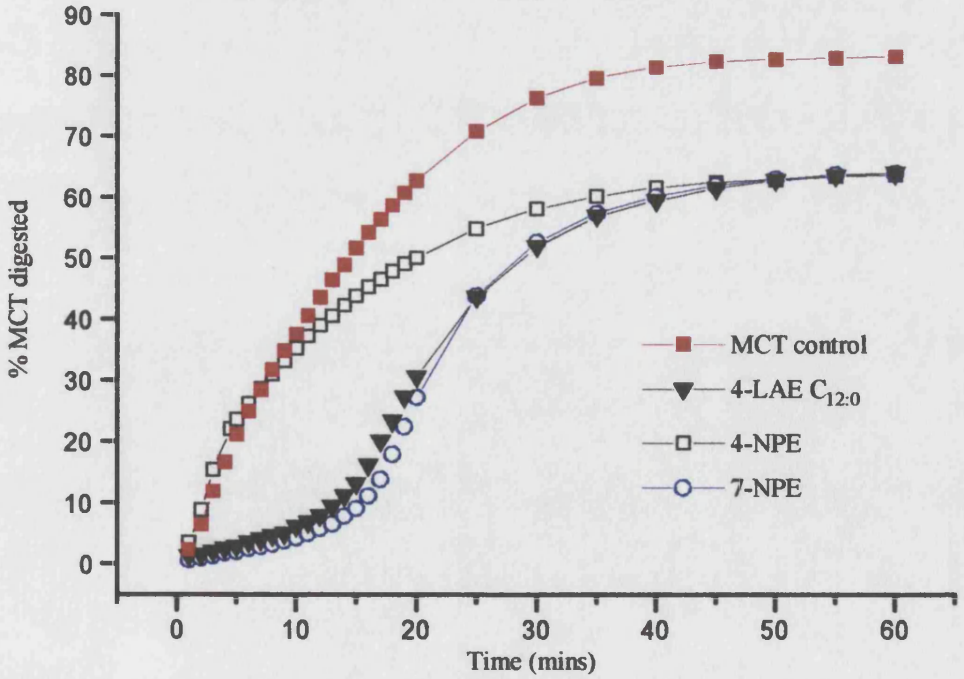


Figure 4.15c Comparison of pancreatin activity towards MCT (1 g) in the presence of alcohol ethoxylated surfactants (1 g) with ethoxylation numbers from 10 to 12.

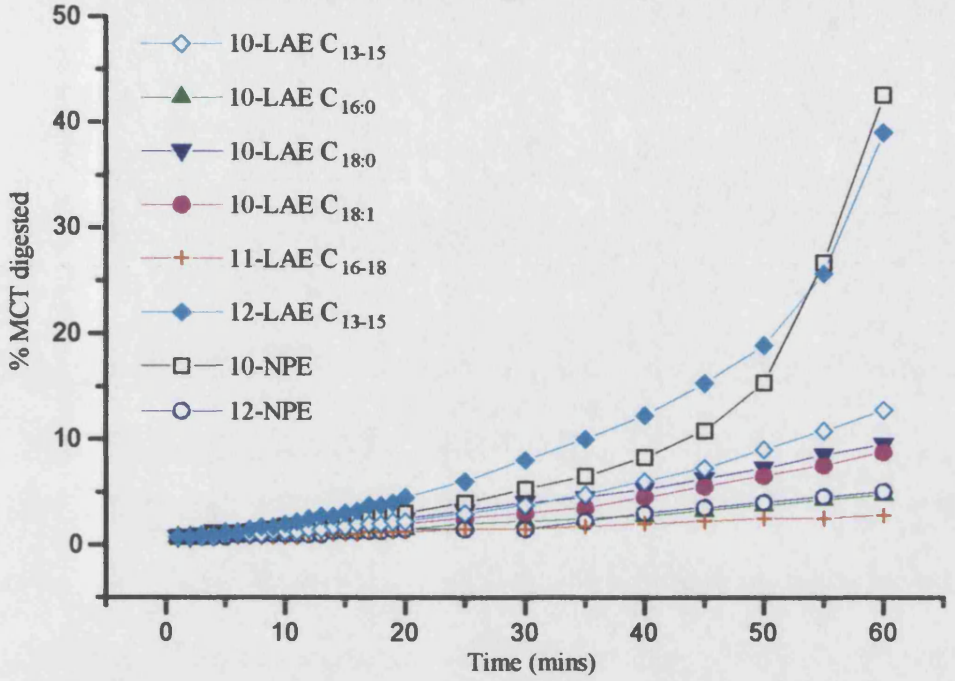


Figure 4.15d Comparison of pancreatin activity towards MCT (1 g) in the presence of alcohol ethoxylated surfactants (1 g) with ethoxylation numbers of 20 and 23.

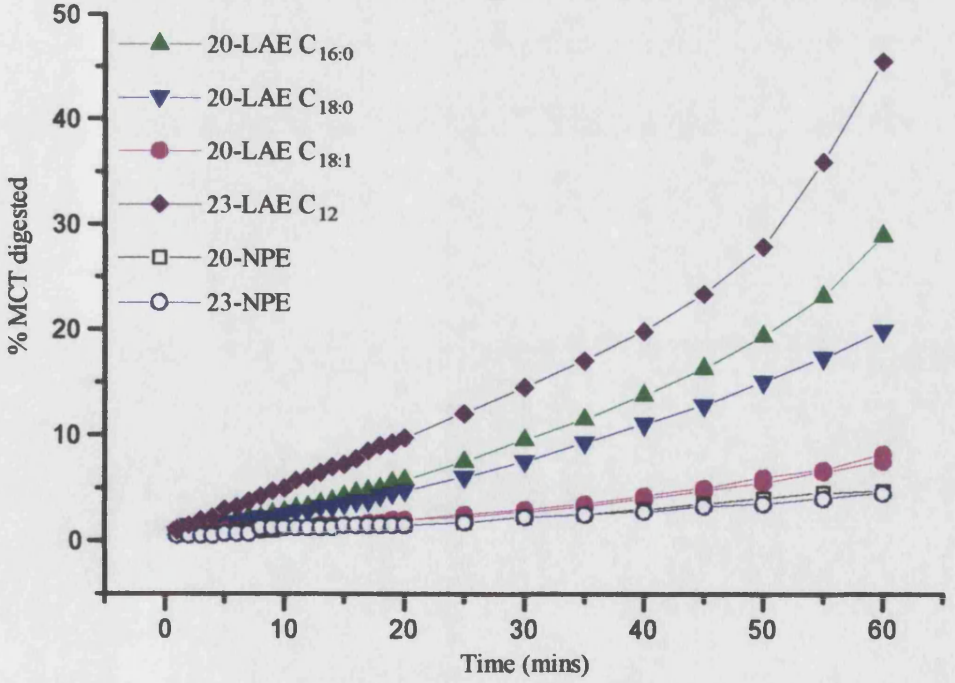


Figure 4.16 $t_{2.5\,\%}$ as a function of surfactant HLB value for nonylphenol ethoxylated surfactants (NPEs) and linear alcohol ethoxylated surfactants (LAEs).

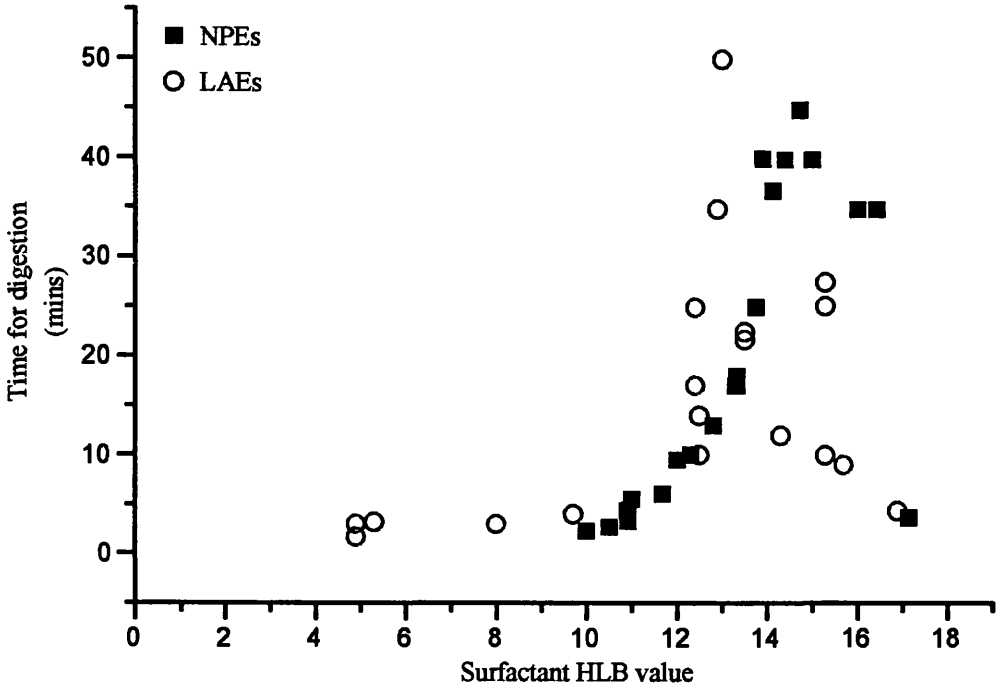


Figure 4.17 Comparison of pancreatin activity towards MCT (1 g) in the presence of castor oil ethoxylates (1 g) (Etocas series).

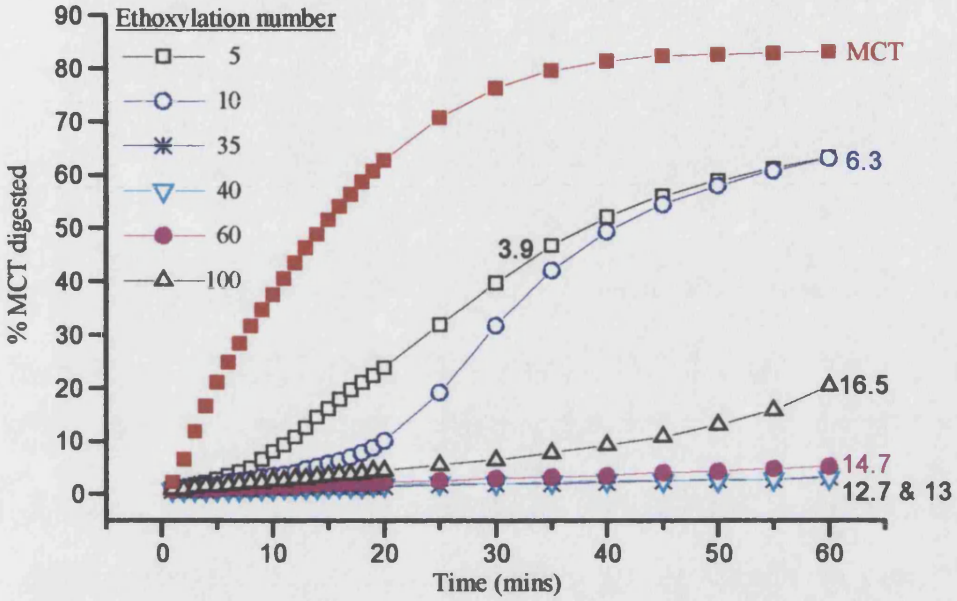
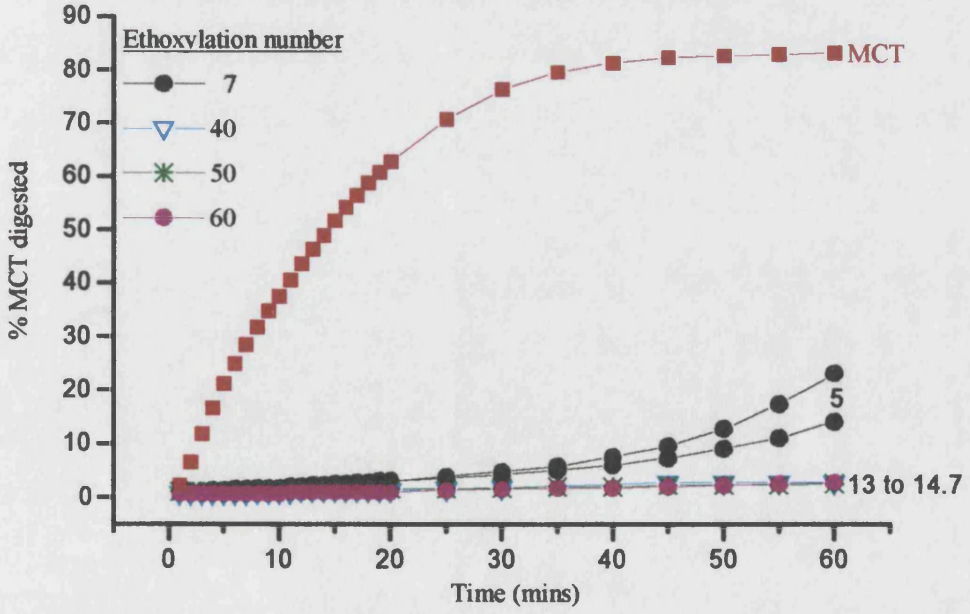


Figure 4.18 Comparison of pancreatin activity towards MCT (1 g) in the presence of hydrogenated castor oil ethoxylates (1 g) (Croduret series).



N.B. HLB values of the surfactants are indicated at the end of the profiles.

Figure 4.19 $t_{2.5\ \%}$ as a function of surfactant HLB value for NPEs and castor oil ethoxylated surfactants (Etocas and Croduret series).

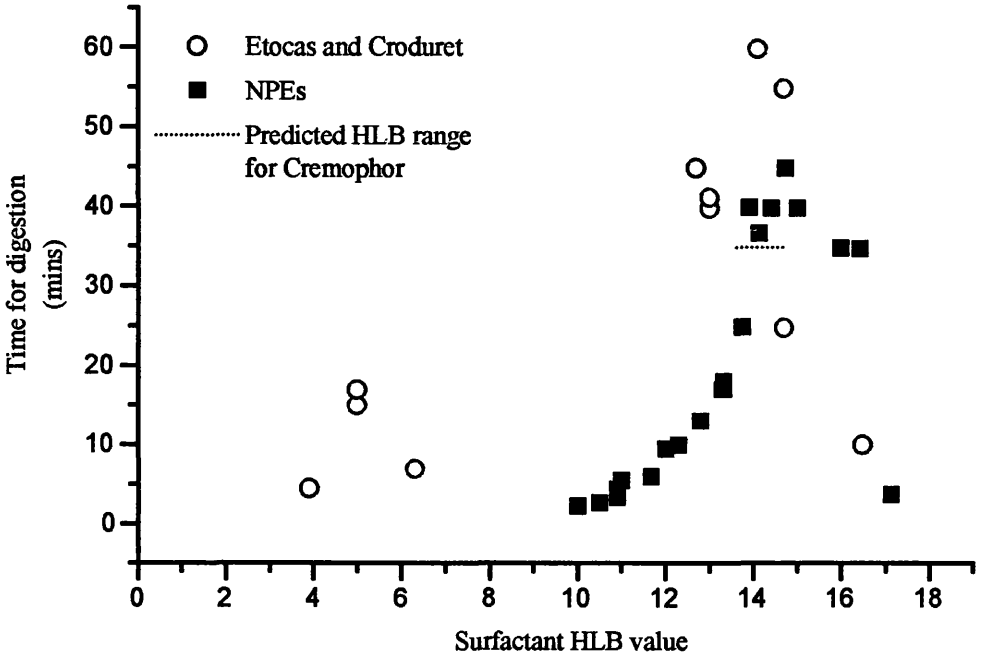


Figure 4.20 Comparison of pancreatin activity towards MCT (1 g) in the presence of sorbitan monoesters (1 g) and PSEs (1 g) with an ethoxylation number of 20.

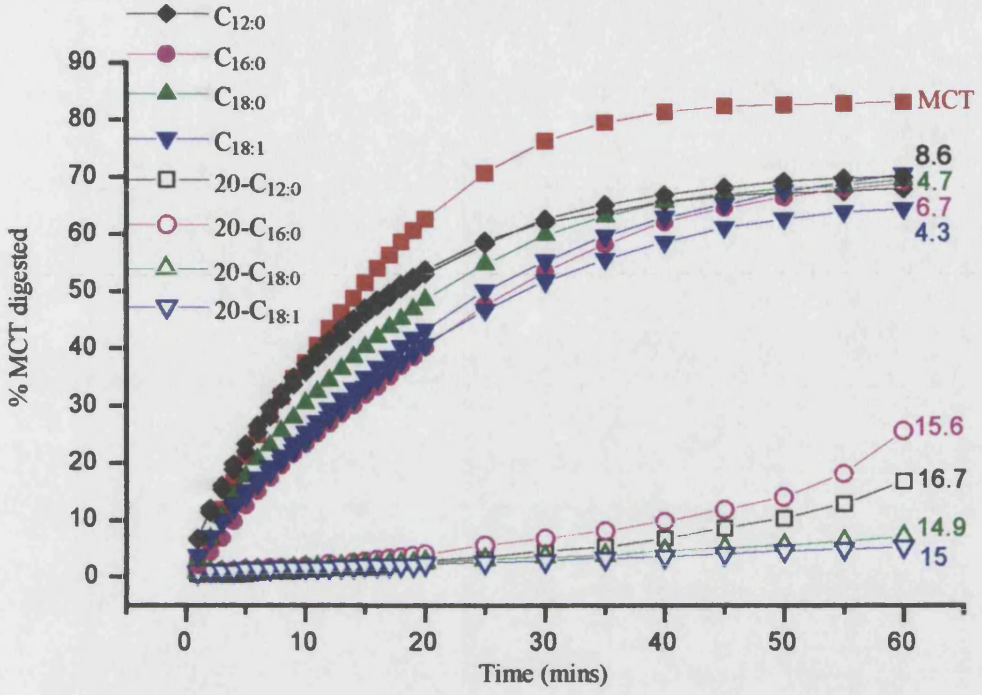
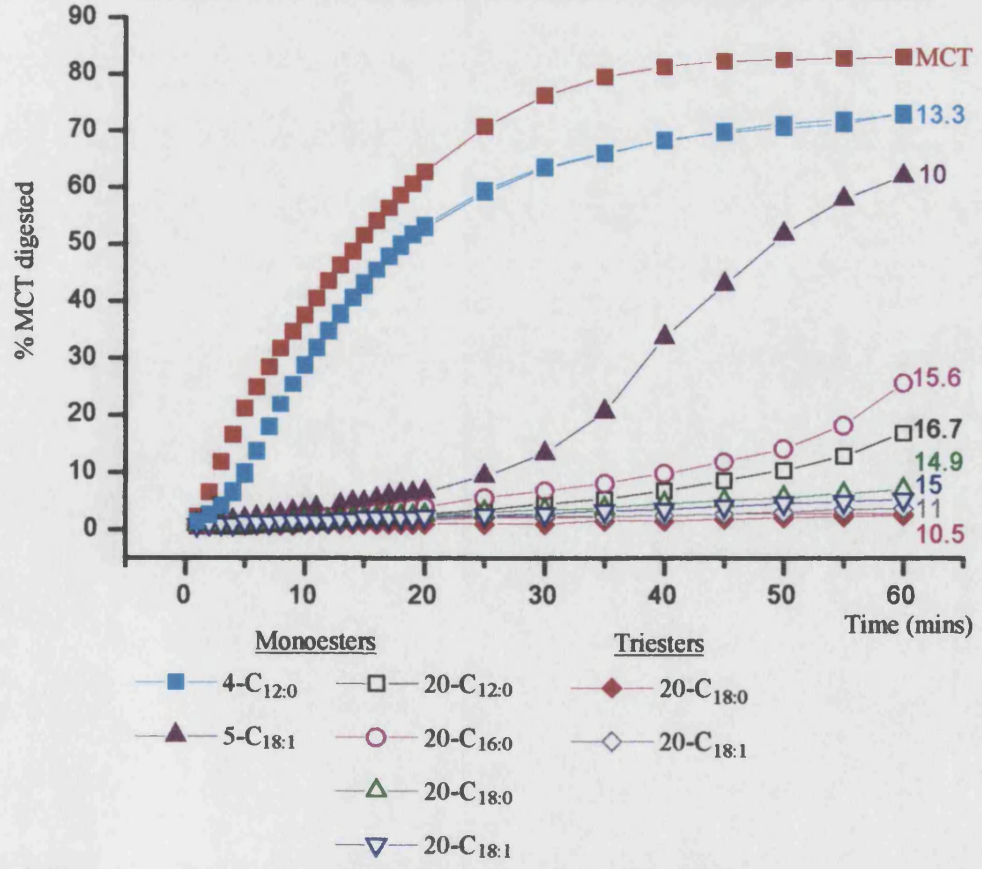


Figure 4.21 Comparison of pancreatin activity towards MCT (1 g) in the presence of PSEs (1 g) with varying extents of ethoxylation and fatty acid esterification.



N.B. HLB values of the surfactants are indicated at the end of the profiles.

Figure 4.22 Comparison of pancreatin activity towards MCT (1 g) in the presence of sorbitan esters (1 g) with 1 (mono), 1.5 (sesquio) and 3 (tri) fatty acids.

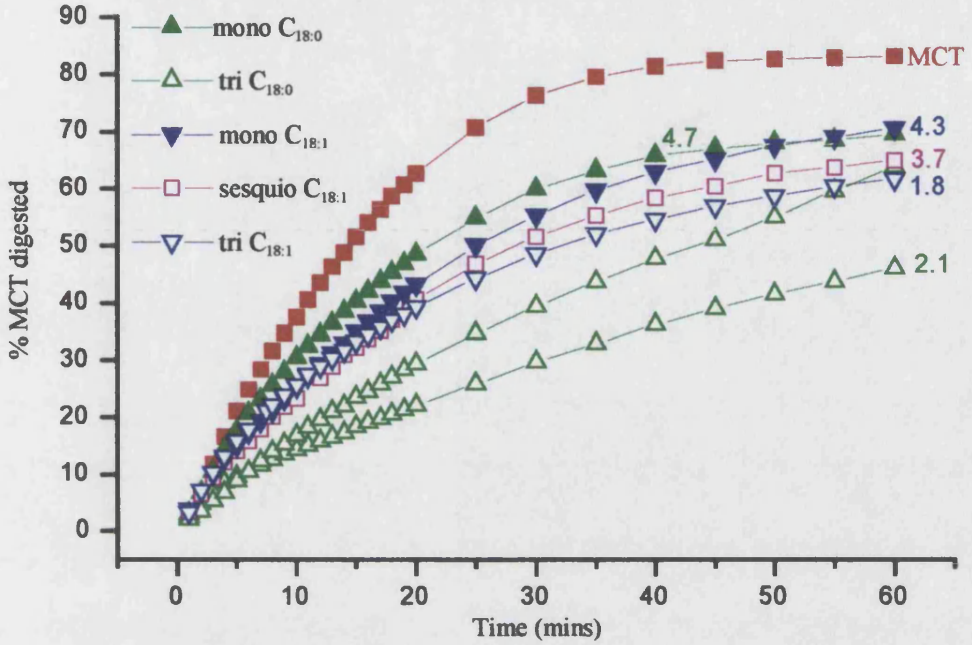


Figure 4.23 Comparison of pancreatin activity towards MCT (1 g) in the presence of polyglycolized glycerides (1 g) (Labrafil series, Labrafac and Labrasol).

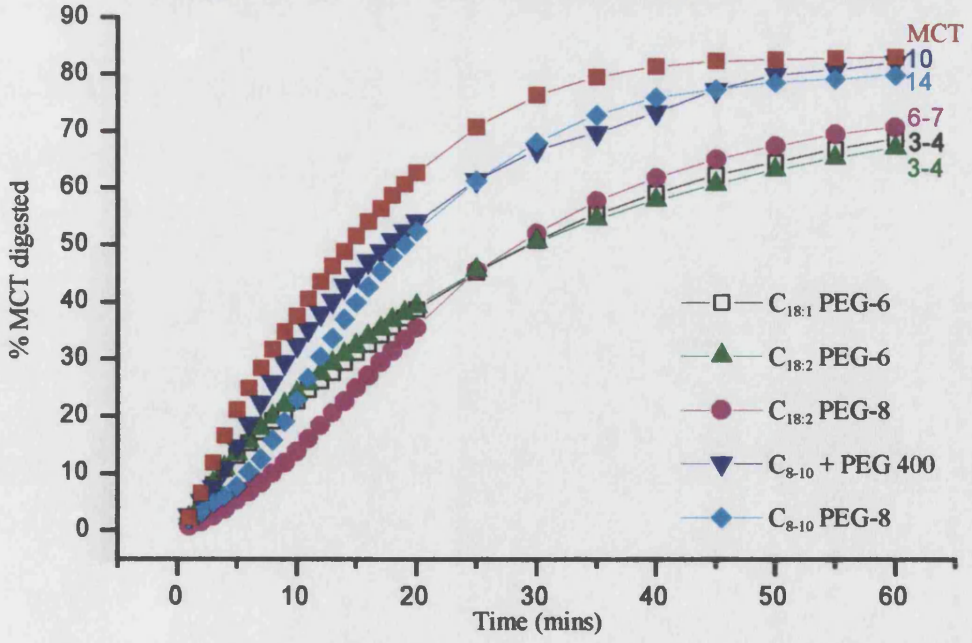


Figure 4.24 A typical ^1H NMR (399.78 MHz) spectrum of a nonylphenol ethoxylated surfactant (sample 10-NPE) in $CDCl_3$ with assignments indicated.

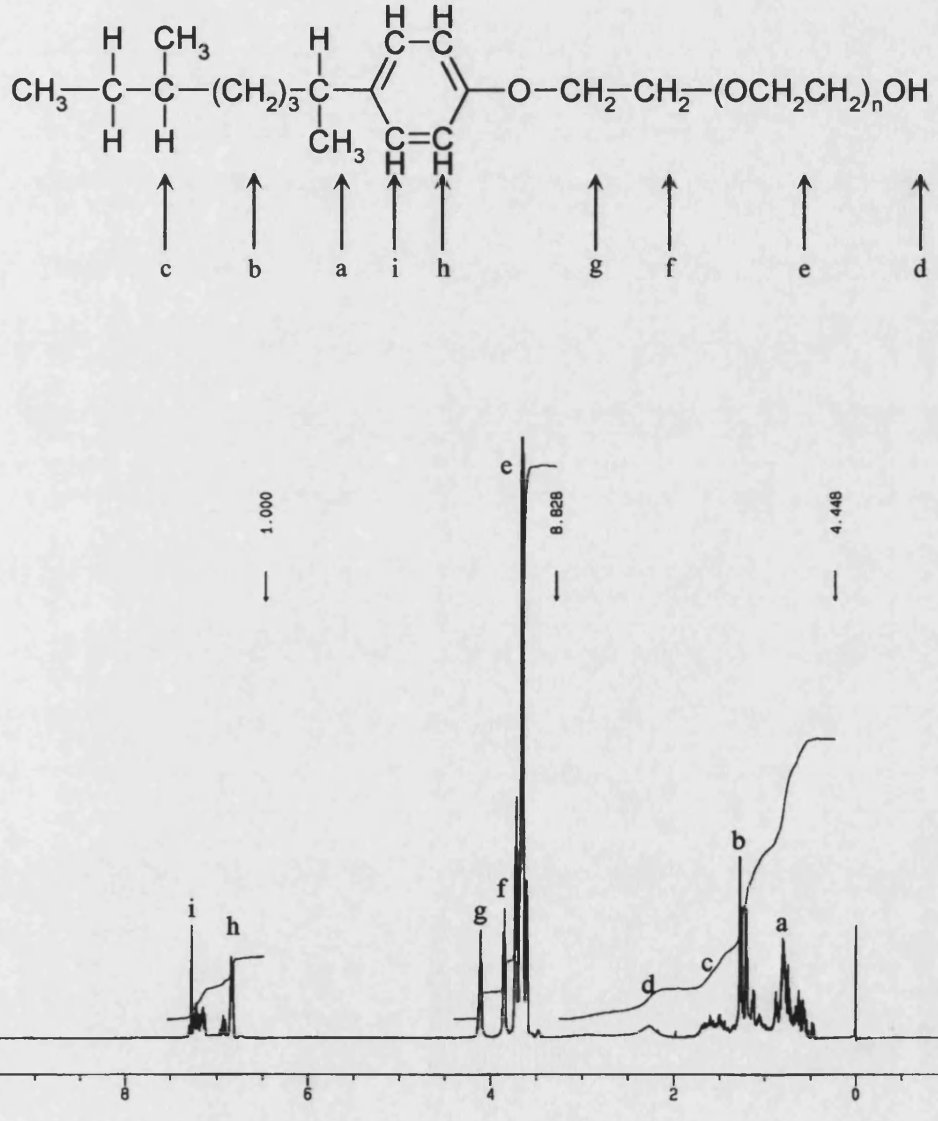
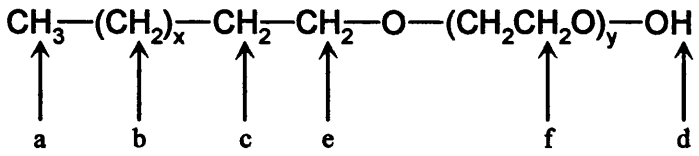


Figure 4.25 A typical ^{1}H NMR (270.16 MHz) spectrum of a linear alcohol ethoxylated surfactant (sample 11-LAE) in $CDCl_3$ with assignments indicated.



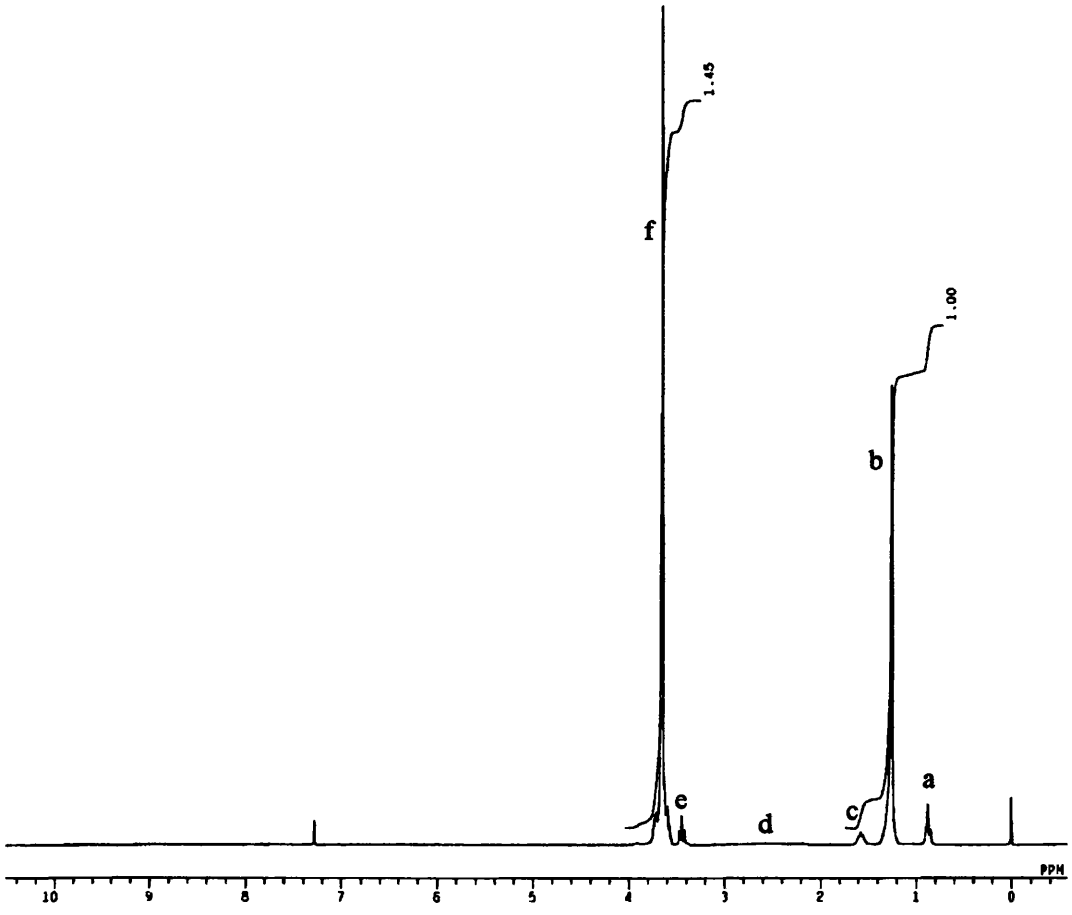


Figure 4.26a $t_{5\%}$ as a function of surfactant HLB value for NPEs using HLB values determined from the ethoxylation number quoted by the manufacturer.

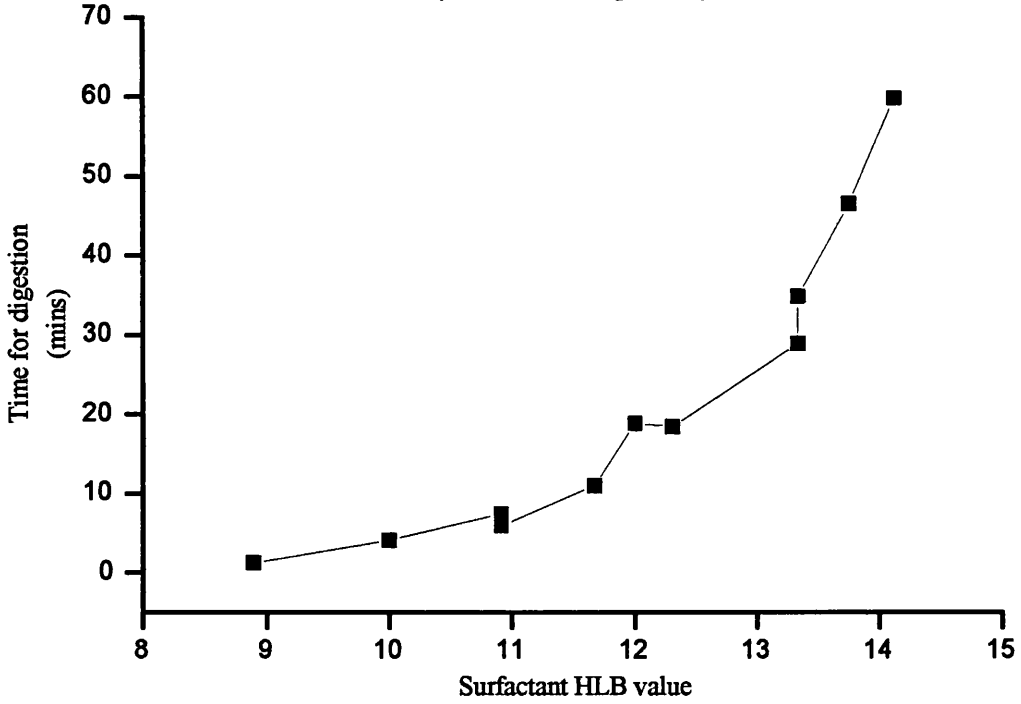
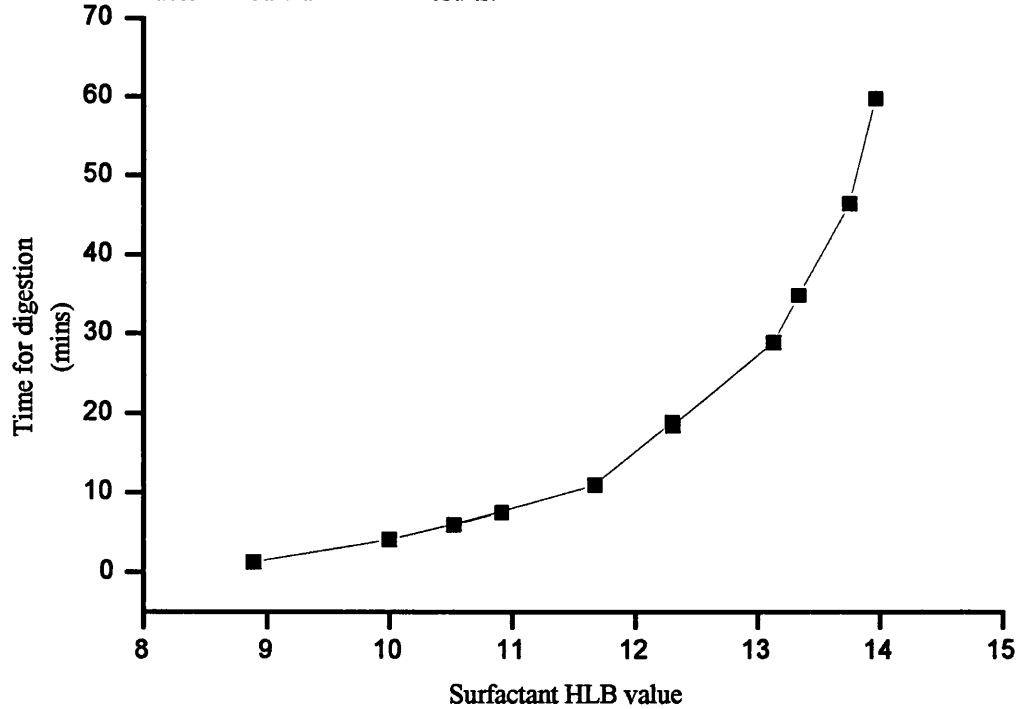


Figure 4.26b $t_{5\%}$ as a function of surfactant HLB value for NPEs using HLB values determined via ^1H NMR results.



Chapter 5 - Issues related to the inclusion of non-ionic surfactants in lipid-based formulations.

5.1 Introduction

At present there are few examples of lipid-based formulations in common usage, despite the suggested advantages of this approach to the bioavailability of hydrophobic drugs[1]. Several reasons have led to this situation, not least of which are of a commercial nature. To produce a lipid-based formulation specialized machinery and expertise is required, possibly involving the need for a contractor, whereas these are normally available in house for conventional tablet formulations. A lipid-based system generally requires development on an individual basis for each drug which may be time consuming and costly. Lack of models to test the efficacy of the formulation and sparse knowledge regarding stability, *in vivo* processing and physiological effects of lipid-based formulations has also restricted their usage[1].

Lipid-based formulations have been proposed to confer an advantage to hydrophobic drug absorption by several mechanisms (see section 1.3), most of which relate to the ability of pancreatic lipase to digest lipid in the formulation. Digestion is suggested to gradually release drug, previously partitioned in the lipid phase, into the GI fluid together with digestion products. These products, in combination with physiological amphipathic molecules and any surfactant present, could form a solubilizing phase in which drug may remain in a dissolved state until absorption. In addition digestion could improve dispersion of the lipid-based formulation, thus providing a higher surface area for drug release.

In an effort to overcome issues such as poor solubility of drugs in simple lipid solutions or to improve the initial dispersion of lipid in GI fluid, lipid-based formulations may contain surfactants and co-solvents. These excipients could have an adverse effect on drug bioavailability from a lipid-based formulation. Possible problems involve alteration in the physical state of the drug upon release from the formulation or the availability of the formulation to lipolytic enzymes. The experimental work in this chapter was thus performed to investigate the influence of surfactants on drug bioavailability from lipid-based formulations.

5.1.1 Self-emulsifying drug delivery systems (SEDDS).

Formulation of SEDDS

Although presentation of a hydrophobic drug dissolved within a lipid-based formulation could be advantageous, the success of this approach may depend upon the dispersibility of the formulation when administered. Ideally the formulation would be a self-emulsifying drug delivery system. SEDDS are isotropic mixtures of oil, surfactant, drug and possibly co-solvent which form fine oil in water emulsions in aqueous media under conditions of limited agitation, similar to those encountered in the GI tract[188]. The large interfacial area of the excellent dispersion formed by SEDDS upon release into the gastric fluid promotes diffusion of dissolved drug from the oily droplets into the surrounding aqueous environment. The drug is therefore presented in a state ready for rapid absorption.

SEDDS have certain advantages over the use of conventional lipid-based formulations such as emulsions. Emulsions are inherently difficult to formulate with high stability characteristics, give poor dosage precision and usually require a high volume to dose ratio[1]. Formulation of a SEDDS with a high solvent capacity for a drug provides a low volume dosage form which may be encapsulated within a hard or soft gelatin shell. The resulting physically stable unit dosage form has the advantages of containing a known concentration of drug in a form acceptable to the patient.

The mechanism by which SEDDS spontaneously emulsify has been widely investigated, with the conclusion that in order to achieve self-emulsification ultra low interfacial tension and / or interfacial disruption is required[188]. Early investigations suggested the speed of emulsification was related to the formation of a liquid crystal phase at the interface upon initial dilution of the oil / surfactant mixture with water[189]. Further support for this has been given by the observation of a relationship between good self-emulsification and the formation of a low viscosity liquid crystalline phase upon addition of a small amount of water to an oil / surfactant mixture[190]. Self-emulsification was suggested to be a result of dynamic formation of liquid crystalline units at the interface promoting further water penetration into the oily phase down aqueous channels. The resulting increase in surface pressure would lead to interfacial disruption and dispersion of the oil as droplets into the bulk aqueous phase[190]. The

relatively insoluble nature of surfactant in water would indicate surfactant is likely to remain in contact with the oil or disperse as a separate emulsion phase[188].

An alternative mechanism of self-emulsification, described as 'diffusion and stranding', has been suggested for SEDDS which include a co-solvent[188]. Rapid migration of co-solvent from the formulation into the bulk aqueous phase is proposed to carry and solubilize some of the water insoluble material from the formulation with it in the process. Upon depletion of co-solvent any remaining oil from the formulation would disperse into droplets.

In theory the use of SEDDS for oral administration of hydrophobic drugs would appear advantageous however few exist; a significant limitation to their use being the effort involved in optimization of a SEDDS. An ideal SEDDS should disperse into an emulsion consisting of droplets < 5 μm in diameter, to reduce the diffusion path of the drug from the droplet[191]. In addition the polarity of the droplets, which is determined by the surfactant present, should promote partitioning of the drug into the aqueous phase[191]. The optimum polarity value for drug release will be related to the oil / water partition coefficient of the drug.

The literature provides some guidelines to aid formulation of a SEDDS. Particle size determination and phase diagrams are recommended to maximize self-emulsification and minimize the diameter of the droplets formed. These parameters are temperature dependent, therefore assessment should be performed at the intended temperature of use i.e. 37°C. A simple combination of MCT with an ethoxylated non-ionic surfactant comprising > 25 % of the formulation can produce a SEDDS[188]. The HLB value of the selected surfactant or blend of surfactants (a blend tends to produce a more stable system), should be between 8 to 18 for an oil in water microemulsion. The choice of surfactant is limited by toxicity profiles, however previous assessment of the self-emulsifying behaviour of a range of non-ionic surfactants revealed unsaturated ester based surfactants to be more effective dispersants in combination with MCT than saturated ether based equivalents[190]. Oleates with a HLB value of approximately 11 formed superior dispersions, which was proposed to be due to the fluidity of the oleate chains in these surfactant molecules[190]. Polyglycolyzed glycerides have also been demonstrated to be efficient emulsifiers for SEDDS[191]. A recent study[192] stated that the use of a surfactant with a HLB value in the range of 10 to 15 will optimize

self-emulsification, especially if in combination with a co-emulsifier such as a medium chain (C_8-C_{10}) monoglyceride. Other excipients used to formulate lipid microemulsions have been listed by Constantinides[193].

The choice of surfactant can have an impact on the release of drug from a SEDDS by affecting solubilization capacity, a property maximized by the use of hydrophobic surfactants which do not migrate. However to raise the solubility of drug in a SEDDS a hydrophilic surfactant or co-solvent may be required. This can be counterproductive in terms of improving drug dissolution as the hydrophilic material may rapid diffuse (via the 'diffusion and stranding' mechanism) from the SEDDS into the aqueous phase. Precipitation of the drug may then occur, especially if the drug has a low log P (i.e. 2-3)[188].

In more general terms liquid formulations of SEDDS are to be preferred over waxy pastes as they are easier to assess in terms of the morphology of the drug[188]. The number of excipients is best kept to a minimum as with all formulations. Even a slight alteration in the final formulation of a SEDDS (such as addition of co-solvent and / or drug[194, 195]) can result in the loss of self-emulsifying behaviour. This is due to the sensitivity of self-emulsification to the oil / surfactant ratio. In some cases inclusion of drug can be advantageous resulting in a decrease in droplet diameter[1]. Formulation of SEDDS is discussed more fully in a recent review by Pouton[188].

Bioavailability of drugs from SEDDS.

The profile of SEDDS as a possible delivery method has been raised by the use of microemulsions for delivery of the peptide, cyclosporin A (cyclosporin)[195]. The pharmacokinetics of cyclosporin, a highly lipophilic polypeptide of fungal origin, have been reported as widely variable when administered by the oral route[196] and altered absorption has been related to the presence of bile, food and the formulation used[197]. The original formulation used by Sandoz for cyclosporin, (Sandimmune®) consists of oil and alcohol in combination with polyglycolyzed glycerides and is available as a solution and encapsulated in soft gelatin capsules[193]. It is described as a self-emulsifying system although *in vitro* tests reveal a crude emulsion[198]. When Sandimmune® solution was administered to rats as per dosage directions (median droplet diameter 4 μm) or after homogenization (median droplet diameter 2 μm) the

homogenized form increased blood levels nearly 2-fold. This suggested that the bioavailability of cyclosporin from an emulsion could be improved by reducing the size of the emulsion droplets[198].

An absolute bioavailability of around 30 % has been stated for Sandimmune® although this value has been reported to vary from 7 to 90 %[196]. Sandimmune Neoral® (Neoral), described as a microemulsion pre-concentrate, was therefore developed in an attempt to optimize the bioavailability of cyclosporin. Neoral is composed of lipids (corn oil mono-, di- and triglycerides), surfactant (polyoxyl-40 hydrogenated castor oil), hydrophilic co-solvent (propylene glycol) and antioxidant (DL-tocopherol). Upon dilution of Neoral in the GI fluid a monophasic oil in water microemulsion is readily produced[199].

Comparison of the bioavailability of cyclosporin from the Sandimmune® and Neoral formulations has revealed the presence of fat[200] or bile[201] to increase bioavailability from the Sandimmune® capsule. Bioavailability from Neoral capsules was much less sensitive to the presence of fat, although in the case of bile a minimal dependence was indicated. These results suggest the coarse emulsion formed by Sandimmune® requires further dispersion via the *in vivo* mechanisms of lipid processing whereas the Neoral microemulsion has inherent dispersion characteristics mainly unaffected by the physiological environment.

Neoral is now generally the preferred formulation of cyclosporin due to a reduction in inter and intra-patient variation, thus providing a more consistent plasma concentration with time profile compared to Sandimmune®[202]. Neoral has also been demonstrated to exhibit linear pharmacokinetics[203], which provides a means of dosage titration with increased accuracy when compared to the original Sandimmune® formulation.

Further *in vivo* studies are required to demonstrate the efficacy of SEDDS in humans. Some evidence of the potential ability of SEDDS to improve drug bioavailability above levels shown by other delivery systems has been provided by animal studies. A SEDDS formulation (comprised of MCT and non-ionic surfactant) and a PEG 600 solution formulation were employed to administer a lipophilic anti-viral compound (Win 54954) to beagle dogs[204]. Absolute bioavailability did not differ between the two formulations due to substantial first pass metabolism however plasma profiles from

the SEDDS displayed more consistent absorption with time. This was proposed to be a function of the inherent solubilization and high quality dispersion produced by SEDDS rather than a feature of the lipid component or use of a solution dosage form; a conclusion based also upon the erratic plasma profiles obtained for the same drug when administered in a soybean oil solution.

Four different formulations (a tablet, a suspension, a PEG 600 solution and a SEDDS) of a benzodiazepine derivative, (L-365 260) with low aqueous solubility were administered to dogs[205]. The SEDDS and PEG formulations both increased relative bioavailability and C_{max} values to levels 7 to 8 times higher than those shown by the formulations where L-365 260 was not dissolved. For L-365 260 bioavailability appeared to be improved by a formulation which eliminated the dissolution stage. A further study compared the bioavailability of a highly lipophilic naphthalene derivative, Ro-150778 from three different formulations (a SEDDS, a PEG 400 solution and a tablet)[191]. When these were administered to non-fasted dogs the SEDDS gave four times the relative bioavailability of the PEG solution which in turn was 15-fold greater than the tablet.

These results illustrate that for a drug with limited aqueous solubility it is advantageous to use a formulation where the drug is in solution, such as a PEG solution or a SEDDS, thus enabling avoidance of the dissolution stage. When the aqueous solubility of a drug is very low, drug may precipitate upon release of a PEG solution into the aqueous environment of the GI tract. The same effect would not be expected from SEDDS as these have the capacity to solubilize drug and hold it in a dissolved form within the aqueous environment. A further advantage of SEDDS is their tendency to give more reproducible plasma profiles than seen with PEG solutions. This could possibly be accounted for by the rapid dispersion and gastric emptying of the formulation in the GI tract. The resulting high drug plasma levels likely to be achieved shortly after dosage of a SEDDS will require consideration if the drug in question has a narrow therapeutic index[188].

Overall the use of SEDDS appears advantageous to enhance the bioavailability of poorly water-soluble drugs, whether solely by avoidance of the dissolution stage or in combination with solubilization and rapid dispersion.

5.1.2 Outline of this investigation.

SEDDS have excellent dispersion behaviour which removes the need for digestion to enhance solubilization of drug. This is not the case for the lipid-based formulations previously discussed in this thesis, which appear reliant upon the lipid digestion cascade (a process also known as lipolysis) to enhance bioavailability of drug. For these formulations digestion can release drug from the lipid phase into a solubilized phase formed from the components of bile and lipolytic products.

Digestion of these lipid-based formulations and subsequent release of drug from the lipid will be enhanced if the surface area of the lipid droplets is increased. This is frequently accomplished by the use of a mixture of hydrophilic surfactant (classified here as a HLB of > 10) with lipophilic surfactant (HLB ≤ 10). The lipophilic surfactant is necessary to initiate crude emulsification of the oil allowing the hydrophilic surfactant to produce a fine and uniform oil in water emulsion. Drug absorption from emulsions of this nature is thought likely to be faster and more consistent compared to a situation where drug is concentrated within large oil droplets.

However inclusion of surfactants within a lipid-based formulation could be disadvantageous due to the ability of many non-ionic surfactants to inhibit digestion of lipid (as demonstrated by *in vitro* studies in Chapter 4). This chapter aims to examine further the influence of surfactants upon digestion of lipid-based formulations. In general terms the aim was to develop two lipid-based formulations. One of the formulations was to be available for digestion by pancreatin whereas the other was not. The difference in the availability of the two formulations to pancreatin was to be brought about by the informed selection of surfactants for inclusion in the formulations. The whole procedure was to be performed for two different drugs. The resulting formulations were intended for an *in vivo* study to determine if inhibition of lipid digestion by surfactants would impair drug bioavailability.

All experimental work described in this chapter was performed during a period of secondment at R P Scherer at their premises in Swindon. The overall intention was to investigate whether the observations reported in Chapters 3 and 4 were general phenomena which could lead to intellectual property of general significance. Prior to commencing this work the following statements represented the available knowledge:

- Natural lipolysis of triglyceride within the GI tract can enhance the dissolution rate of a hydrophobic drug co-administered with triglyceride.
- Evidence from *in vitro* studies demonstrated the addition of a hydrophilic surfactant to triglyceride can in some cases totally inhibit lipolysis.
- The inhibition of lipolysis due to hydrophilic surfactants can be significantly reduced by certain lipophilic surfactants.

The preliminary investigations in this chapter (sections 5.2-5.3) were designed to assess a range of excipients, namely non-ionic surfactants, available for use in lipid-based formulations. Knowledge gained was to be employed to develop several lipid-based formulations. These formulations were required to test the virtues of a new class of lipid delivery systems, described below as consisting of:

1. A hydrophobic drug
2. A medium or long chain triglyceride capable of undergoing digestion in the presence of pancreatic lipase under *in vivo* conditions. The triglyceride was intended to be a solvent for the drug in addition to providing a source of lipolytic products upon digestion.
3. A hydrophilic surfactant or mixture of hydrophilic surfactants able to disperse the triglyceride upon *in vivo* administration. These are expected to inhibit the *in vivo* lipolysis of triglyceride.
4. A lipophilic surfactant or mixture of lipophilic surfactants capable of reducing inhibition of lipolysis due to the hydrophilic surfactant. Lipophilic surfactants preferred for this purpose being fatty acids and mono and / or di-glycerides of fatty acids.

In vivo tests were planned to compare drug bioavailability from such a formulation described above, with bioavailability from a similar system unable to undergo lipolysis. In addition it was proposed that both lipid-based formulations were to be compared with a current marketed product of the drug in question.

Two drugs were selected for the investigation on the basis of high hydrophobicity and poor absorption from conventional solid dosage forms due to dissolution rate limitation. These were fenofibrate, a broad spectrum lipid-modulating agent and cinnarizine, an antihistamine commonly used for vestibular disorders and motion sickness. The objective was to develop two formulations for each drug which were identical apart from the identity of the lipophilic surfactant used. The lipophilic surfactants were to be selected in order that one formulation was able to undergo lipolysis whereas the other was not.

The lipid-based formulations required for the *in vivo* tests were made to the following specifications. The final unit dosage form was to be a soft gelatin capsule containing a total 1 g of formulation. The formulation was to be comprised of drug within a lipid / surfactant carrier system. The intended composition of the formulation is described below with points 2 to 5 referring to the carrier system:

1. A drug (between 0.1-50 % $^{w}/_{w}$ of the final formulation)
2. A triglyceride to comprise 10-90 % $^{w}/_{w}$ (preferably 25-45 %) of the formulation. A percentage of the triglyceride may be replaced with a digestible lipophilic surfactant.
3. A hydrophilic surfactant to comprise 10-60 % $^{w}/_{w}$ (preferably 30-45 %) of the formulation at a concentration which gave adequate dispersion and also substantially inhibited *in vivo* lipolysis of the triglyceride.
4. A hydrophilic solvent to comprise 0.1-20 % $^{w}/_{w}$ of the formulation, if required, to increase drug solubility.

5. A lipophilic surfactant to comprise 5-60 % $^{w}/_{w}$ (preferably 20-40 %) of the formulation:

   For the lipolysing carrier system

   A lipophilic surfactant capable of substantially reducing the inhibitory effect of the hydrophilic surfactant.

   For the non-lipolysing carrier system

   A lipophilic surfactant unable to overcome the inhibitory effect of the hydrophilic surfactant.

The remainder of this chapter describes the stages involved in development of these formulations for fenofibrate and cinnarizine, followed by their evaluation under *in vitro* and *in vivo* conditions.

Section 5.2 describes the assessment of a range of lipophilic surfactants for ability to reactivate pancreatin activity within a previously non-lipolysing system (comprised of a digestible triglyceride and a hydrophilic surfactant). This approach provided basic information to aid development of the formulations by identifying surfactants capable of reactivation of lipolysis. Investigations in section 5.3 assessed the solubility of the drugs within possible formulation constituents and enabled selection of possible combinations of excipients exhibiting miscibility and suitable characteristics of self-dispersion.

Results from these preliminary investigations enabled selection of promising formulations (section 5.4) which underwent assessment and modification to ensure compliance with specifications for lipolysing and non-lipolysing formulations.

Following selection of the lipolysing and non-lipolysing formulations, *in vitro* models representing gastric dispersion and digestion in the duodenum were used to assess drug release from the respective formulations (section 5.5). Finally the formulations were prepared for *in vivo* testing by a contractor. The *in vivo* results are discussed in section 5.6.

5.1.3 Materials

In addition to materials listed in Chapters 2 and 3

Hydrophilic Surfactants

Trade name	Description	HLB	Supplier
Crillet 1	20-ethoxy sorbitan monolaurate	16.7	Croda
Crillet 2	20-ethoxy sorbitan monopalmitate	15.6	Croda
Crillet 4	20-ethoxy sorbitan mono-oleate	15.0	Croda

Lipophilic Surfactants

Trade name	Description	HLB	Supplier
Etocas-5	5-ethoxy castor oil ethoxylate	3.9	Croda
Imwitor 375	Glyceryl citrate/lactate/linoleate/oleate	N/A	Hüls
Imwitor 988	Caprylic/capric glycerides	N/A	Hüls
Lauroglycol	Propyleneglycol monolaurate	4-5	Alfa Chemicals Ltd.
Maisine 35-1	Glycerol mono-oleate	4	Alfa Chemicals Ltd.
Marlowet R11	11-ethoxy castor oil ethoxylate	6.8	Hüls
Neobee M20	Propylene glycol dicaprylate (65 %) /caprate (35 %)	10	Stepan
Plurol oleique	Polyglyceryl-6-dioleate	10	Alfa Chemicals Ltd.
Priolene 6930	C_{14}-C_{20} fatty acids (69-75 % oleic acid)	N/A	Unichema
Span 20 5571	Sorbitan monolaurate	8.6	Surfachem
Span 80 0599	Sorbitan mono-oleate	4.3	Surfachem

(N/A = HLB value not available)

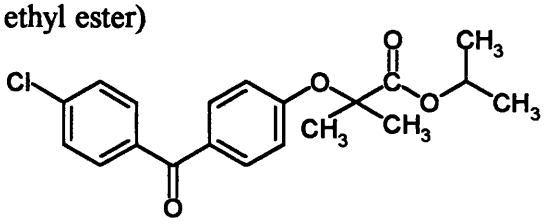
Other materials

Ethanol absolute	(HPLC grade)	E-0665 Fisons
Capric acid	99 % n-decanoic free acid	C1875 Sigma
Caprylic acid	99 % n-octanoic free acid	C2875 Sigma
Monocaprate	1-monodecanoyl-rac-glycerol ($C_{10:0}$)	M2140 Sigma
Monocaprylate	1-monocapryloyl-rac-glycerol ($C_{8:0}$)	M2265 Sigma
Monoolein	1-monooleoyl-rac-glycerol ($C_{18:0,\,[cis]-9}$)	M7765 Sigma
Oleic acid	95 % free acid	O1630 Sigma

Drugs

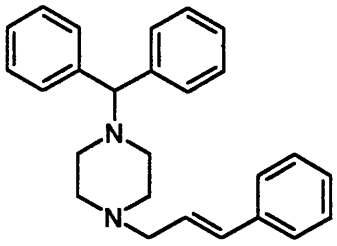
Fenofibrate — F6020 Sigma

(2-(4-{4-Chlorobenzoyl} phenoxyl)-
2-methylpropanoic acid 1-methyl
ethyl ester)



Cinnarizine — C5270 Sigma

(1-cinnamyl-
4-diphenylmethylpiperazine)



(Address of suppliers listed in Chapter 4)

5.2 Study of the apparent ability of lipophilic non-ionic surfactants to reactivate pancreatin activity.

Experimentation by colleagues at R P Scherer[108] had indicated that inclusion of lipophilic surfactants within a system where digestion of triglyceride was inhibited by hydrophilic surfactant allowed pancreatin activity to proceed. This observation suggested that the presence of lipophilic surfactant changed the nature of the reaction system so as to allow pancreatin to hydrolyze the triglyceride substrate.
Certain lipophilic surfactants inherently contain as part of their composition partial products of triglyceride digestion. This fact, in combination with the proposal that lipolytic products released during digestion may be able to reactivate a surfactant inhibited system, (see section 3.3.6), immediately suggests a possible mechanism for the above observation.
In the following study several different classes of non-ionic lipophilic surfactants were examined for the ability to reactivate pancreatin activity towards MCT in a system inhibited by hydrophilic surfactant. Information thus obtained was subsequently compared with the ability of pancreatin to utilize the lipophilic surfactants as substrate. This approach was taken to enable the measured reactivation of lipolysis to be related to the digestion of triglyceride and / or the lipophilic surfactant for the purpose of elucidation of the mechanism of reactivation.

5.2.1 Investigation of the ability of pancreatin to hydrolyze lipophilic surfactants.

A range of non-ionic surfactants with dominant lipophilic character (HLB≤ 10) were selected to undergo the standard pH-stat assay of lipolysis (section 2.2) in the absence of triglyceride. This was to determine if pancreatin could utilize the surfactants as substrate and if so to what extent.

Method

A digestion profile was produced (in duplicate) for each surfactant listed in section 5.1.3 under conditions of the standard pH-stat assay. In each case 1 g of surfactant was added to simulated bile solution and stirred well before commencement of the

assay. Titrant volume was recorded at intervals of 10 minutes over an assay duration of 120 minutes. The digestion profiles represent the quantity of alkali required to neutralize digestion products released from the surfactant by pancreatin against time.

Results

Digestion profiles of each lipophilic surfactant are shown in Figures 5.1a-b together with a control profile of MCT (1 g). This was included for comparison of titrant volumes from surfactant with levels obtained when using a natural triglyceride substrate. The duplicate digestion profiles of each surfactant were almost identical in all cases therefore each surfactant is represented by a typical profile in Figures 5.1a-b. Background pancreatin activity in the absence of triglyceride was known to occur within the standard pH-stat assay (section 2.8) due to enzyme activity upon lecithin, a component of simulated bile solution. It was therefore necessary to ensure the digestion recorded on each profile did not include any enzyme activity due to the presence of lecithin.

To enable subtraction of the blank rate due to lecithin from each lipophilic surfactant profile, a blank digestion profile of pancreatin acting upon simulated bile solution was performed in duplicate. Titrant recordings were taken at identical time intervals to those used for the lipophilic surfactants. A typical blank rate profile of simulated bile solution was selected for subsequent subtraction from each lipophilic surfactant profile and the MCT profile before their representation in Figures 5.1a-b. This approach allowed any enzyme activity which may not be due to digestion of surfactant to be discounted. The blank rate profile used is illustrated in Figures 5.1a-b for comparison.

Digestion profiles from the surfactants were then divided according to whether the rate of digestion shown on the profile exceeded that shown by the simulated bile solution blank profile for the complete assay period. Figure 5.1a includes digestion profiles from surfactants which displayed a rate of digestion below the blank profile for at least part of the assay period. Surfactants illustrating an extent of digestion above the blank profile throughout the total assay period are illustrated in Figure 5.1b. The digestion profiles from the ethoxylated castor oil surfactants, Etocas-5 and Marlowet R11 (Marlowet) shown in Figure 5.1a exhibited negative values after

deduction of the blank profile. This result suggested that digestion of lecithin present in the assay system was inhibited in addition to the surfactant being unavailable to pancreatin as a substrate.

The sorbitan monoesters (Figure 5.1a) have been digested to a limited extent, the C_{12} version (Span 20) appearing to be more available to pancreatin than the $C_{18:1}$ (Span 80) version. From the structure of these surfactants (section 4.9) it could be suggested that pancreatin is acting by cleavage of fatty acid from the sorbitan moiety.

A limited extent of digestion was also exhibited on the digestion profile of Plurol oleique (Figure 5.1a). This surfactant is described as a polyglyceryl ester of polymer units, each of which is composed of six glycerol molecules incorporating a $C_{18:1}$ di-glyceride. Cleavage of the $C_{18:1}$ fatty acids from the di-glyceride would appear to be the site of hydrolytic activity of pancreatin upon Plurol oleique.

The digestion profile of oleic acid (Priolene) showed slight digestion (Figure 5.1a) over the two hour assay period. This is difficult to explain but may relate to the purity of the oleic acid source used, described by the manufacturer as a $C_{14\text{-}20}$ fatty acid with an oleic acid content of 69 - 75 %. No further information was available regarding other possible contaminants.

The profiles of the propylene glycol esters (Neobee M20 (Neobee) and Lauroglycol) showed significant digestion demonstrating that fatty acid esterified onto a propylene glycol backbone is readily available for hydrolysis by pancreatin (Figure 5.1b). Lauroglycol, a C_{12} monoester showed less digestion than Neobee, a $C_{8/10}$ diester, which is to be expected due to the higher number of fatty acids available per molecule of Neobee. The shorter fatty acid chain may also be easier for pancreatin to hydrolyze. Further evidence for increased hydrolysis with shorter fatty acid chains is given by the results from Maisine (Figure 5.1b), a $C_{18:1}$ monoglyceride. This surfactant exhibited less digestion than the C_{12} propylene glycol monoester, despite the natural glyceryl substrate backbone.

Highest digestion was shown by Imwitor 988, a partial glyceride of $C_{8\text{-}10}$ described as consisting of 50 % monoglyceride, 40 % diglyceride and 6 % triglyceride (Figure 5.1b). This result was to be expected as Imwitor 988 contains the natural substrate of pancreatin together with partial digestion products.

Imwitor 375 is a partially neutralized ester of mono and diglycerides of linoleate ($C_{18:2}$) and oleate ($C_{18:1}$) with citric and lactic acid. It contains 20 % of both mono and diglycerides (fatty acid chains of oleate and linoleate) and less than 2 % triglyceride with the remaining 58 % being composed of complex esters with citric and lactic acid. From the low rate of digestion seen (Figure 5.1b) the citric and lactic acid esters are probably unavailable to pancreatin activity, with the longer chain C_{18} fatty acids possibly slower to digest than the C_{8-10} of Imwitor 988.

Overall the results in Figures 5.1a-b demonstrate the castor oil ethoxylates to be unavailable for lipolysis by pancreatin. The extent to which the other surfactants were digested appeared to depend upon the number of ester bonds per molecule with fatty acids. The chain length of the fatty acid may also be involved with results from the surfactants with medium chain fatty acids indicating higher availability to pancreatin than the long chain equivalents. A noticeable influence due to the nature of the ester backbone, i e propylene glycol, glyceryl or polyglyceryl was not observed.

5.2.2 Quantification of the reactivation of pancreatin activity by lipophilic surfactants.

To quantify the ability of lipophilic surfactants to reactivate pancreatin activity when incorporated in a previously inhibited system comprised of MCT and a hydrophilic surfactant, a non-lipolysing model of triglyceride digestion was required.

Cremophor at a concentration of 1 g was already known to efficiently inhibit pancreatin activity towards 1 g of MCT (Figure 3.12a) when digested under conditions of the standard pH-stat assay. In addition a digestion profile of Cremophor (1 g) had demonstrated the inability of pancreatin to digest the surfactant (results not shown). Cremophor (1 g) was therefore considered ideal in combination with MCT (1 g) to create a model of a non-lipolysing system, with both substrate and surfactant virtually unavailable for lipolysis.

Each lipophilic surfactant tested in section 5.2.1 was included within the non-lipolysing model of triglyceride digestion. The reactivation of lipolysis observed is discussed in terms of the percentage of total lipolytic activity which could be related directly to digestion of the triglyceride.

Method

Digestion profiles were produced (in duplicate) under conditions of the standard pH-stat assay for each lipophilic surfactant when included in the non-lipolysing model. Substrate and surfactant mixtures were prepared by directly weighing 1 g of MCT, 1g of Cremophor and 1 g of the lipophilic surfactant under examination into the same glass vial. The three constituents were mixed by vortexing to ensure homogeneity before addition to the simulated bile solution.

Titrant volume was recorded at intervals of 10 minutes over an assay duration of 120 minutes. The digestion profiles represented the volume of titrant necessary to neutralize digestion products released with time.

Results

The resulting digestion profiles were classified into two groups depending upon whether the extent of digestion in the presence of lipophilic surfactant exceeded or was less than that shown by the non-lipolysing model.

The lipophilic surfactants unable to reactivate lipolysis within the two hour assay period were the castor oil ethoxylates (Etocas-5 and Marlowet), propylene glycol esters (Lauroglycol and Neobee) and a polyglyceryl ester (Plurol oleique). The digestion profiles from these surfactants were below that shown by the non-lipolysing model and are not illustrated.

An explanation of the inability of these surfactants to reactivate lipolysis may involve the structure of the surfactant. The castor oil ethoxylates have already been demonstrated in Chapter 4 to be inhibitory towards pancreatin, irrespective of ethoxylation number, and in Figure 5.1a to be unavailable for digestion by pancreatin. It is likely that these low ethoxylated castor oils have increased the non-lipolysing layer upon the triglyceride droplets preventing lipolysis.

The situation is different for the propylene glycol esters which when digested alone were available for hydrolysis by pancreatin (Figure 5.1b). Cremophor and triglyceride present in the system has in some manner rendered the propylene glycol esters inaccessible to pancreatin. They may be partitioned in the core of the lipid droplets with MCT, shielded from pancreatin activity by an outer hydrophilic layer of Cremophor.

The digestion profiles from the lipophilic surfactants capable of reactivating pancreatin activity in the non-lipolysing model are shown in Figure 5.2. Also shown is the profile from the non-lipolysing model and a control profile of MCT (1 g), for comparison of the extent of reactivation achieved. The surfactants capable of reactivating lipolysis, to varying extents, included the sorbitan monoesters (Span 80, Span 20), oleic acid (Priolene), monoglyceride (Maisine) and the partial glycerides (Imwitor 988 and 375). Only Imwitor 988 gave a digestion profile which showed a rate and extent of digestion similar to the MCT control profile (Figure 5.2).

The ability of surfactants composed of partial triglyceride digestion products (Maisine, Priolene and Imwitor) to reactivate enzyme activity in the non-lipolysing model may be explained by the following hypothesis. When the MCT and surfactants are added to the simulated bile solution the digestion products inherent in the surfactant could be assumed to accumulate at the interface of the MCT droplets, in the same manner as would occur during normal lipolysis due to their physicochemical characteristics. The result of this could be an increase in colipase partitioning (see section 3.3.6) into the interface, with a consequent increase in lipase bound to the substrate. Enzymatic activity would increase, further lipolytic products would accumulate and eventually 'bud off' the lipid droplet, clearing away some of the Cremophor molecules in the process.

The C_{12} sorbitan monoester (Span 20) showed a surprising ability to reactivate lipolysis (Figure 5.2) compared to the availability of this surfactant to pancreatin activity when digested alone (Figure 5.1a). There was a lag period of approximately 50 minutes before inhibition from Cremophor was overcome and then lipolysis proceeded at a rapid rate. Initial hydrolysis of the sorbitan-fatty acid ester bond may be responsible for overcoming inhibition by providing a source of lipolytic products. The lag phase may reflect the difficulty with which pancreatin hydrolyzes Span 20.

The $C_{18:1}$ sorbitan monoester (Span 80), was much less effective at reactivating pancreatin activity compared to Span 20 (Figure 5.2). The increase in the length of the fatty acid chain increases the lipophilicity of the material. The presence of a double bond in the fatty acid chain has already been suggested to enhance the ability of surfactant to partition into lipid compared to saturated equivalents (section 4.7.5). The combined influence of these properties has probably resulted in Span 80

partitioning into the lipid interface to an extent where the fatty acid ester bond is unavailable to surface activity from pancreatin. The limited concentration of fatty acids released may lead to a higher rate of enzyme activity with time however within the assay period of 120 minutes the effect of Span 80 is negligible compared to the other surfactants illustrated (Figure 5.2).

Overall these results tend to suggest that reactivation of lipolysis is due to partial digestion products, present as part of the composition of the lipophilic surfactant or released from the surfactant by the hydrolytic activity of pancreatin. If pancreatin activity is necessary to release digestion products from the lipophilic surfactant a lag phase is seen on the profiles. This phase probably relates to the time taken for digestion products to increase to a level where colipase partitioning into the interface is enhanced.

Further explanations for the reactivation of lipolysis may involve alteration in the nature of the emulsion system upon addition of lipophilic surfactant to the non-lipolysing model. The addition of a lipophilic surfactant which combines well with MCT to the non-lipolysing model may result in a higher interfacial area. The proportion of Cremophor present may no longer be sufficient to form a barrier over the whole interfacial area, thus allowing some initial hydrolysis to occur.

Addition of a lipophilic surfactant to the non-lipolysing model will change the HLB of the system, which may in turn affect the nature of the emulsion. This could result in dissociation of Cremophor from the triglyceride and subsequent reactivation of pancreatin activity due to exposure of the lipid interface. Further surfactants need to be tested to determine if a relationship exists between the HLB value of a lipophilic surfactant and the degree of reactivation achieved.

5.2.3 How much of the apparent reactivation of enzyme activity in the non-lipolysing model is due to digestion of MCT ?

Lipophilic surfactants have been proposed to reactivate digestion within the non-lipolysing model by increasing the accessibility of triglyceride substrate to pancreatin. The lipolysis recorded (Figure 5.2) could however be due to pancreatin activity upon

a variety of substrates present in the reaction mixture. These include triglyceride, Cremophor, lipophilic surfactant and lecithin within the simulated bile solution. Contribution to the profiles in Figure 5.2 from the digestion of Cremophor and lecithin was thought unlikely, as a digestion profile of Cremophor with the blank rate due to lecithin removed consisted of negative values across the whole profile. For this reason the simulated bile solution blank profile was not subtracted from the digestion profiles in Figure 5.2. Thus by elimination, digestion within the reactivated non-lipolysing model could be due only to pancreatin activity upon the MCT substrate and / or the lipophilic surfactant.

In most cases the lipophilic surfactants able to reactivate lipolysis were surfactants which included partial digestion products of triglyceride (Maisine, Priolene, Imwitor 375 and 988). It would not therefore be surprising if the additional digestion seen was a result of hydrolysis of the lipophilic surfactant by pancreatin.

To determine the proportion of reactivated lipolysis which could be attributed to pancreatin activity upon MCT, the lipophilic surfactant profiles (Figures 5.1a-b) were compared with profiles from the same surfactant when included in the non-lipolysing model. The profiles were compared by examination of the total volume of titrant used upon completion of the assay. The total volume of titrant recorded on the digestion profile of the lipophilic surfactant alone was converted into a percentage of the total volume of titrant recorded from the same surfactant included in the non-lipolysing model. This approach gave the percentage of reactivated digestion which could be attributed as solely due to pancreatin activity upon MCT and results are shown in Table 5.1.

Lipophilic Surfactant	Description of surfactant	% of reactivated digestion attributable solely to digestion of MCT
Priolene	Oleic acid	93
Span 20	C_{12} sorbitan monolaurate	91
Imwitor 375	Glyceryl citrate/lactate /linoleate/oleate	89
Maisine	Glycerol mono-oleate	80
Span 80	$C_{18:1}$ sorbitan mono-oleate	69
Imwitor 988	Caprylic/capric glycerides	44

Table 5.1 Percentage of reactivated pancreatin activity which could be attributed solely to digestion of MCT.

From Table 5.1 the presence of oleic acid in the non-lipolysing model increased the extent of digestion of MCT to higher levels than those shown by the other lipophilic surfactants listed, however all of the surfactants increased digestion to a level above that shown when the same surfactant was digested alone. The percentage of reactivated digestion is described as that attributable solely to the digestion of MCT. This is based on the assumption that the lipophilic surfactant is digested to the same extent when present alone as in the non-lipolysing model. However this is not necessarily the case as the propylene glycol and polyglyceryl classes of surfactants (section 5.2.2) have been demonstrated to be less available to pancreatin in the non-lipolysing model. Likewise it is not possible to be certain that the model would not increase the extent to which a lipophilic surfactant is digested.

To investigate further the possible source of digestion recorded in these reactivated systems a series of experiments were performed to compare the extent of digestion of MCT and Imwitor 988 when digested together and individually. Separate digestion profiles were produced under the conditions of the standard pH-stat assay for MCT (1 g), Imwitor 988 (1 g), MCT (1 g) combined with Imwitor 988 (1 g) and MCT (1 g), Imwitor 988 (1 g) and Cremophor (1 g) (i.e. the reactivated non-lipolysing model). All of the resulting profiles are displayed in Figure 5.3 with the same information shown for Imwitor 375 in Figure 5.4.

From examination of Figure 5.3 it can be seen that the amount of digestion which occurred upon individual digestion of MCT and Imwitor 988 was not additive when the two were digested in combination. This suggests that the presence of Imwitor 988

as an emulsifier with MCT makes one or both substrates less available to pancreatin than when simply dispersed alone in simulated bile solution. Alternately the effect could be a result of the high level of digestion products released (as indicated by the final titrant volume of 4.5 ml). The concentration of bile salt available in the *in vitro* model may have been insufficient to solubilize and effectively remove the digestion products from the system. This may have inhibited further enzyme activity; a situation which would not occur if the same combination was digested *in vivo*.

When the same series of experiments were repeated for Imwitor 375, (a partial glyceride less available for hydrolysis than the 988 version) the MCT and Imwitor combination was digested to the same extent as the reactivated non-lipolysing model (Figure 5.4). However for both profiles the rate and extent of digestion was lower than that seen when the same quantity of MCT was digested alone. This suggests that the presence of Imwitor 375 resulted in MCT being less available to pancreatin. Accumulation of digestion products was probably not responsible for this effect as higher titrant volumes were recorded for MCT when digested alone. A possible explanation could involve the formation of a complex, unavailable to pancreatin activity, between MCT and the glycerol citric and lactic acid esters of Imwitor 375. These experiments did not provide any further information regarding the proportion of reactivation which may be due to MCT digestion. However in the case of Imwitor 375 the amount of MCT and Imwitor digested in the non-lipolysing model reached the same extent as the same quantities with no Cremophor present, confirming that all of the inhibitory effect due to Cremophor was eventually overcome.

5.3 Preliminary considerations necessary for formulation of the lipid-based systems.

The preliminary investigations contained in this section were designed to rationalize selection of possible combinations of the three main components of the lipid-based formulations. These components were to be a triglyceride, a hydrophilic surfactant known to be inhibitory towards pancreatin activity upon triglyceride and a lipophilic surfactant. The lipophilic surfactant may or may not be able to overcome the said

inhibition depending upon the specification of the formulation in terms of lipolysing behaviour.

The solubility of fenofibrate and cinnarizine in various micellar solutions of triglyceride digestion products and surfactants was to be determined. This was to enable selection of components for the formulation which were most beneficial in terms of enhancing solubility of the drugs, both within the formulation and the presumed solubilized phase *in vivo*. In addition various combinations of triglyceride, hydrophilic and lipophilic surfactants, described as formulations, were to be assessed for miscibility and ease of dispersion; both important characteristics of the final formulation.

5.3.1 Drug solubility in micellar solutions.

Assessment of drug solubility in various micellar solutions was intended to enable selection of formulation constituents favourable to drug dissolution. If the triglyceride and surfactants selected enhanced drug solubility this may be advantageous upon release of the formulation from the capsule into the stomach. The drug would be less likely to precipitate upon release into the aqueous gastric fluid, thus remaining partitioned in the lipid and / or dissolved in a solubilized phase formed by the formulation ready for absorption.

The solubility of fenofibrate and cinnarizine was determined in micellar solutions of triglyceride digestion products and selected surfactants. In each case the micellar solutions were prepared in simulated bile solution to increase representation of the possible solution formed upon release of the formulation into the intestine.

Appropriate combinations of monoglycerides and fatty acids were used to represent long and medium chain triglyceride digestion products. If the solubility of a drug was shown to be improved by either long or medium chain products this would indicate which type of triglyceride to select for use in the formulation. The lipolytic products released upon digestion of triglyceride in the formulation would then have a high solubilization potential for the particular drug.

Method

The solubility of fenofibrate and cinnarizine was determined in the following micellar solutions:

Micellar solution	Total concentration
• Long chain lipolytic products to represent soya bean oil (oleic acid 11 mM, monoolein 5 mM)	0.5 % $^{w}/_{v}$
• Medium chain lipolytic products to represent fractionated coconut oil (caprylic acid 11 mM, monocaprylate 5 mM, capric acid 7 mM, monocaprate 3.5 mM)	0.5 % $^{w}/_{v}$
• Cremophor	0.5 % $^{w}/_{v}$
• Crillet 4	0.5 % $^{w}/_{v}$
• Simulated bile solution	
• Tris-maleate buffer pH 6.5	

The simulated bile solution and Tris-maleate buffer were prepared as per formulas given in section 2.2.1. To prepare the remaining solutions the appropriate quantities of constituents for each solution were added to simulated bile solution followed by processing in an ultrasonic bath at 37°C for 1 hour to ensure homogeneity. Each solution was then adjusted to pH 7.5 using a pH-stat and titrant of 1 M NaOH or 0.1 M HCl as appropriate.

Saturated solutions of drug were prepared in triplicate for each solution and left to reach equilibrium solubility in an ultrasonic bath for 1 hour at 37°C. The solutions were mixed by vortexing and 2 ml of the resulting suspension passed though a filter (Whatman P.V.D.F 13mm Syringe filters, 0.2 μm Cat. No. 6779-1302) to remove any drug not dissolved; the first 0.5 ml of filtrate being discarded to allow for any drug-filter binding which may occur.

The concentration of drug dissolved in the resulting filtrate was determined via a high-performance liquid chromatographic (HPLC) method developed and performed for

each drug by the analytical division of R P Scherer. Sample solutions underwent HPLC analysis within a 24 hour period of preparation.

Results

The mean solubility of fenofibrate and cinnarizine in each solution is represented in Figures 5.5a and b respectively (with the standard deviation from 3 replicates indicated). All of the micellar solutions can be seen to have increased drug solubility levels above those shown in the buffer solution, included to represent aqueous solubility.

Figures 5.5a-b show both drugs to be preferentially soluble in the long chain rather than the medium chain lipolytic product solution. Fenofibrate and cinnarizine exhibited higher solubility in the micellar solutions of surfactants than in the medium chain lipolytic product micelles, with highest solubility overall determined in the Cremophor solution. Both drugs were soluble within the simulated bile solution whereas solubility within buffer could not be determined with accuracy due to the trace concentrations present.

Overall the results demonstrate the same pattern of solubility enhancement of fenofibrate and cinnarizine across the range of micellar solutions tested. For cinnarizine solubility values varied widely from zero up to 29 μg / ml whereas fenofibrate exhibited a narrower solubility range from zero to 6.5 μg / ml. Careful selection of constituents would appear prudent when devising the formulations for fenofibrate, to aid enhancement of the extremely low drug solubility. Choice of constituents for the formulations of cinnarizine could be considered to be less critical.

5.3.2 Miscibility Studies upon potential formulations.

Knowledge of the behaviour of triglyceride, hydrophilic and lipophilic surfactant when combined together was necessary to aid selection of a formulation which remained miscible after encapsulation within a soft gelatin capsule. Any phase separation of constituents after the encapsulation stage could result in drug recrystallization and thus the addition of a drug dissolution stage upon release of the formulation from the capsule.

To examine the miscibility of triglyceride, hydrophilic and lipophilic surfactant when present in different proportions, use was made of a three-component phase diagram. Each of the axes on the diagram represented the percentage contributed to the formulation by each of the three constituents. The effect of varying the proportions of constituents present upon the miscibility of the formulation could thus be examined. Eight combinations of triglyceride, hydrophilic and lipophilic surfactant were selected to provide a wide range of possible formulations, necessary as ideally the lipid-based formulation for each drug was to contain different constituents to widen the scope of any intellectual property which resulted from the study. Comparison of the different combinations was also intended to reveal effects from a particular constituent in terms of ease of dispersion and miscibility.

The possible formulations contained either Crillet 4, a polyethoxylated sorbitan mono-oleate or the hydrogenated castor oil ethoxylate, Cremophor, as the source of hydrophilic surfactant, both of which were known to be efficient inhibitors of pancreatin. The triglyceride chosen was either fractionated coconut oil (FCO) representing a medium chain triglyceride or soya bean oil (SBO) as an example of a long chain triglyceride.

Several different lipophilic surfactants already demonstrated (section 5.2.2) to be capable of reactivating lipolysis were used, oleic acid, Maisine (glycerol mono-oleate) and Imwitor 988 (caprylic/capric glycerides), with the latter two also tested in combination. A co-solvent, such as ethanol could be necessary to increase the solubility of drug in the final formulation, therefore examination for any possible influence from ethanol upon miscibility was also made for certain formulations.

Method

The constituents used within each potential formulation (named A to H) are listed in Table 5.2.

Formulation	Hydrophilic surfactant	Triglyceride	Lipophilic surfactant
A	Crillet 4	SBO	Imwitor 988
B	Crillet 4	FCO	Maisine 35-1
C	Crillet 4	FCO	Imwitor 988/Maisine (1:1)
D	Crillet 4	SBO	Imwitor 988/Maisine (1:1)
E	Cremophor	SBO	Oleic acid
F	Cremophor	FCO	Oleic acid
G	Crillet 4	SBO	Oleic acid
H	Crillet 4	FCO	Oleic acid

Table 5.2 Combinations of triglyceride, hydrophilic and lipophilic surfactant selected as possible formulations.

Points were selected evenly distributed across a triangular phase diagram. Each point represented a possible formulation, the percentage of triglyceride, hydrophilic and lipophilic surfactant present within that formulation being specified by the corresponding axes.

For each point the quantities of triglyceride, hydrophilic and lipophilic surfactant necessary to produce 5 g of formulation were weighed into the same 20 ml clear glass vial (accuracy + 0.02g of target weight for each constituent). The mixture was mixed by vortexing and the vial was then left to stand at room temperature for 7 days after which time an assessment was made in terms of phase separation.

If the mixture of constituents formed a continuous phase throughout the vial the formulation was classified as miscible whereas vials which displayed two or more phases were described as immiscible. Maisine at room temperature was not a clear solution therefore when incorporated with other formulation constituents white specks were seen throughout the formulation. Although it would be possible to remove these particles by centrifugation, for this study they were not considered to constitute a phase separation when assessing the formulations for construction of the phase diagrams.

For each vial of formulations A to D where no phase separation was evident after 7 days, absolute ethanol (0.5 ml) was added to the formulation resulting in an

approximate ethanol concentration of 9 % $^{v}/_{w}$. This represented the maximum concentration of co-solvent likely to be used in a formulation as higher concentrations can start to impair the soft gelatin shell. After addition of ethanol the formulations were again thoroughly mixed by vortexing and left to stand for 2 days before reassessment of miscibility status.

Results

For formulations A to H the results are illustrated upon individual phase diagrams (Figures 5.6 a-h). The diagrams indicate the phase state of the formulation when triglyceride, lipophilic and hydrophilic surfactant are present in varying proportions.

Hydrophilic surfactant

From inspection of the phase diagrams (Figures 5.6 e-f) formulations containing the hydrophilic surfactant Cremophor exhibited poor miscibility, irrespective of the type of triglyceride present. The viscosity of the resulting opaque formulations increased in line with proportion of Cremophor present. The oleic acid was observed to be layered upon the surface of the formulation. Formulations E and F containing Cremophor were thus discarded from the study due to the exhibited immiscibility. Their opaque nature was a further disadvantage as this would have prevented visual examination to determine if drug was dissolved.

Formulations containing Crillet 4 (Figures 5.6 a-d, g & h) gave superior results in terms of miscibility compared to Cremophor. In the case of these formulations when immiscibility was observed it appeared to relate to the type and concentration of triglyceride present (see below). Crillet 4 was thus indicated to be the preferred choice of hydrophilic surfactant for use in the final formulations.

Triglyceride

Considered as pairs, formulations C and D and G and H are identical except for the source of triglyceride. Formulation C (Figure 5.6c) remained miscible until FCO concentration was raised to 80 % whereas formulation D (Figure 5.6d) exhibited loss of miscibility at a SBO concentration of as low as 20 %.

The same behaviour was reflected by formulations G and H where formulation G (Figure 5.6g) exhibited loss of miscibility at a SBO concentration of 60 % whereas formulation H remained miscible up to a FCO concentration of 80 % (Figure 5.6h). These results suggest the use of FCO rather than SBO will improve the formulation when miscibility is difficult to achieve.

Lipophilic surfactant

For formulations containing FCO and Crillet 4 the choice of lipophilic surfactant had no influence upon miscibility (Figures 5.6 b, c, & h). When comparing formulations composed of the less miscible SBO with Crillet 4 (Figures 5.6 a, d, & g) the choice of lipophilic surfactant could improve miscibility. From the combinations tested inclusion of oleic acid (Figure 5.6g) allowed formulation of miscible systems at higher SBO concentrations than Imwitor or Maisine (Figures 5.6 a & d).

Absolute ethanol

Results from addition of absolute ethanol to formulations A to D had little effect upon phase behaviour. However the solubility of Maisine did improve in the presence of ethanol, with concentrations of up to 40 % being completely dissolved. Ethanol also promoted miscibility of a formulation when FCO was the source of triglyceride; the previously immiscible formulations B and C containing 80 % FCO became miscible upon addition of ethanol. Due to the lack of adverse effects from inclusion of ethanol in formulations A to D it was not felt necessary to examine the effect of ethanol on formulations E to H at this stage.

5.3.3 Assessment of ease of dispersion of potential formulations.

Ideally to maximize drug absorption the formulations chosen were required to form a fine, uniform emulsion upon release from the capsule. This was to increase the likelihood of even drug dispersion throughout the gastric fluid, with promotion of rapid and consistent gastric emptying into the small intestine. In addition both the lipolysing and non-lipolysing formulations were required to form a dispersion similar in quality, to ensure fair comparison in the *in vivo* study.

For all formulations which exhibited miscibility upon the phase diagrams (Figures 5.6 a-h), characterization of the ease of dispersion was performed using the simulated gastric dispersion model. This model was intended to represent the dispersion formed upon release of the formulation into the aqueous gastric fluid in the stomach.

Method

To prepare Formulations A to H for characterization in terms of ease of dispersion, 1 g (+ 0.02 g) of the formulation mixture was encapsulated into an air fill soft gelatin capsule (17.5 tube 2A) and heat sealed.

The simulated gastric dispersion model involved allowing 1g of encapsulated formulation to emulsify for 30 minutes in 100 ml of simulated gastric fluid (0.34 M NaCl in 0.1 M HCl) contained in a 500 ml round bottomed flask. Agitation to simulate stomach movement was provided by an overhead stirrer. This slowly rotated the flask (30 r.p.m) within a water bath held at 37°C.

After 30 minutes the resulting solution was examined in the flask under a bright electric light source. Using the appearance of scattered light, the emulsion formed was characterized according to the grades listed in Table 5.3.

Grade	Dispersion	Emulsion characteristics
A	Excellent	Translucent micro-emulsion which may be optically clear (droplet diameter 0.01-0.1 μm) or bluish haze (0.1-0.2 μm).
B	Good	A uniform emulsion, slightly opaque, grey or white in colour, possibly with a bluish tint.
C	Crude	A non-uniform emulsion with a highly opaque appearance. Trace of residual oil may be present at the surface.
D	Poor	Large globules of non-emulsified oil with a significant amount of oil present at the surface.

Table 5.3 Description of emulsion characteristics used to score the efficiency of dispersion.

Results

The dispersion grade exhibited by each formulation (A-H) was marked inside the miscibility symbol corresponding to that formulation on the relevant phase diagram (Figures 5.6 a-h). From inspection of the phase diagrams (Figures 5.6 a-h) formulations which differed only in the triglyceride present (FCO or SBO) exhibited

the same dispersion grade when all of the constituents were present in identical proportions. Therefore the type of triglyceride used in a formulation did not affect the ease of dispersion.

Figures 5.6 a-d revealed the influence of lipophilic surfactant upon dispersion of the formulation. Imwitor gave an equal or improved dispersion compared to its use in combination with Maisine or results shown by Maisine alone. Use of oleic acid in a formulation (Figures 5.6 e-h) resulted in the worst dispersion behaviour of all those tested.

5.3.4 Conclusions of the preliminary investigations.

The above investigations provided valuable information to aid selection of constituents for the lipid-based formulations. Fenofibrate exhibited very low aqueous solubility in the micellar solutions of digestion products and surfactants. Therefore careful selection of constituents for the formulations of fenofibrate was required to maximize the solubility of the drug.

The medium chain triglyceride, FCO was more likely to form a miscible system than the long chain triglyceride, SBO if the concentration of triglyceride was above 40 %. Interestingly the choice of triglyceride did not appear to affect the ease of dispersion. The choice of lipophilic surfactant in general did not affect miscibility however oleic acid was able to produce a miscible system with higher concentrations of SBO than other lipophilic surfactants tested. Conversely oleic acid was the least efficient lipophilic surfactant of those tested in terms of creating a good quality dispersion, with Imwitor 988 giving superior results in this respect. Miscible formulations were difficult to achieve with Cremophor therefore Crillet 4 was the preferred choice of hydrophilic surfactant for all formulations. Inclusion of ethanol had minimal impact upon miscibility of a formulation.

5.4 Development of lipolysing and non-lipolysing lipid-based formulations.

For each of the drugs (fenofibrate and cinnarizine) two formulations were required, both of which were to consist of triglyceride, hydrophilic and lipophilic surfactant, possibly in combination with a co-solvent for the drug. The constituents were to be present within the percentage limitations described in section 5.1.2. The lipolysing and non-lipolysing formulations for the same drug were to be identical in all respects, differing only in the lipophilic surfactant present and the resultant lipolysing characteristics.
The lipolysing formulation was required to comply with the conditions of digestion Profile III described below, whereas the non-lipolysing formulation was required to comply with the conditions of Profile IV.

Specifications for the lipolysing behaviour of the final formulations.

Profile I The triglyceride alone in the quantity present in 2 g of final formulation.

Profile II The triglyceride in combination with the hydrophilic surfactant thoroughly mixed before addition to the reaction medium in the quantities present in 2 g of final formulation. For the hydrophilic surfactant to be classified as substantially inhibiting lipolysis, Profile II should exhibit a 50 % reduction in fatty acid production after one hour of digestion compared to Profile I.

Profile III The triglyceride in combination with hydrophilic and lipophilic surfactant thoroughly mixed before addition to the reaction medium in the quantities present in 2 g of final formulation. For the lipophilic surfactant to fulfil the description of overcoming inhibitory effects of the hydrophilic surfactant, Profile III should exhibit at least 50 % of the fatty acid production shown by Profile I after one hour of digestion.

Profile IV The triglyceride in combination with hydrophilic and lipophilic surfactant thoroughly mixed before addition to the reaction medium in the quantities present in 2

g of final formulation. For the lipophilic surfactant to fulfil the description of unable to overcome inhibitory effects of the hydrophilic surfactant, Profile IV should not exceed the extent of digestion exhibited by Profile II.

Profiles I-IV were to be performed under conditions of the standard pH-stat assay, at a pH of 7.5, for a period of 60 minutes.

5.4.1 Development of the final formulations for fenofibrate.

Ideally to increase the scope of any intellectual property resulting from the study one of the drugs was to have a formulation containing long chain triglyceride with the other a medium chain triglyceride. As the micellar solubility studies had already demonstrated poor solubility of fenofibrate when compared to cinnarizine, and higher solubility of both drugs in long chain digestion products, it was felt that fenofibrate would benefit most from formulation within a long chain triglyceride (SBO) system. Examination of phase diagrams from formulations containing SBO (Figures 5.6 a, d, & g) revealed Formulation A (containing SBO, Crillet 4 and Imwitor 988) to give the best dispersion when the three constituents were present in approximately equal percentages. As a possible lipolysing formulation 20 % of SBO with 40 % each of Crillet 4 and Imwitor 988 was selected (referred to in future discussion as formulation A1). This selection was made on the basis of compliance with proportions of constituents specified in section 5.1.2, in addition to good dispersion behaviour and miscibility which was independent of the presence of ethanol at a concentration of 9 % $^{v}/_{w}$.

To ensure formulation A1 complied with the requirements for a lipolysing formulation, a series of digestion profiles (Profiles I, II and III) were produced as described in section 5.4. A digestion profile of the quantity of SBO present within 2 g of formulation A1 (Profile I) is shown in Figure 5.7 together with the digestion profile from 2 g of formulation A1 (Profile III). In addition a profile of SBO and Crillet 4 (Profile II) illustrates the ability of Crillet 4 to inhibit the digestion of SBO.

From examination of Figure 5.7 the addition of Crillet 4 to SBO resulted in an 80 % reduction in fatty acid production after one hour of digestion compared to Profile I.

This was well within the requirements for Profile II which only specified a 50 % reduction. In terms of the ability of the lipophilic surfactant to overcome the inhibitory effects of Crillet 4, Profile III (Figure 5.7) demonstrated that inclusion of Imwitor resulted in a 414 % increase in fatty acid production after one hour of digestion compared to Profile I. This result exceeded the minimum 50 % reactivation specified for Profile III and is a consequence of Imwitor 988 providing another source of substrate in addition to SBO. These profiles (Figure 5.7) therefore demonstrated the suitability of formulation A1 for the lipolysing formulation of fenofibrate.

The non-lipolysing formulation needed to contain the same quantities of SBO and Crillet 4 as formulation A1, with Imwitor 988 substituted with a lipophilic surfactant unable to reactivate lipolysis. Five lipophilic surfactants (Etocas-5, Lauroglycol, Marlowet, Neobee and Plurol oleique), demonstrated in section 5.2.2 as unable to reactivate the non-lipolysing model, were selected as possible surfactants for use in the non-lipolysing formulation of fenofibrate.

By substitution of the quantity of Imwitor 988 present in formulation A1 with each one of the lipophilic surfactants (listed above) in turn, five formulations were prepared. Upon assessment for miscibility as per section 5.3.2, all remained miscible after 7 days and upon inclusion of 9 % $^{v}/_{w}$ ethanol, except for the formulations containing Neobee and Marlowet which were discarded from the study.

The remaining three formulations were tested for lipolysing characteristics by production of a digestion profile (Profile IV) using 2 g of formulation. The resulting profiles revealed formulations containing Plurol oleique and Etocas-5 to fulfil the definition of a non-lipolysing formulation by restricting the degree of lipolysis to that shown by Profile II or below (Profile IV of Plurol oleique is illustrated in Figure 5.7). A final decision between the use of Plurol oleique or Etocas-5 in the non-lipolysing formulation was not taken at this stage (see section 5.4.2).

5.4.2 Dissolution of fenofibrate in the final formulations.

A concentration of 5 % $^{w}/_{w}$ fenofibrate was required in the final formulations for use in the *in vivo* study. It was also necessary for fenofibrate to be well within the solubility limit of the drug in each formulation to reduce the risk of drug recrystallization after

encapsulation. Upon addition of fenofibrate at 5 % $^{w}/_{w}$ to formulation A1 and the non-lipolysing formulations containing Plurol oleique or Etocas-5 the drug dissolved, but only with processing in an ultrasonic bath.

As the drug had not dissolved instantly the use of a co-solvent to raise the solubility of fenofibrate within the formulations was investigated. Ethanol up to a concentration of 7.5 % $^{w}/_{w}$ did not significantly improve drug solubility therefore a short chain triglyceride, triacetin, was also included in the formulation to act as an additional co-solvent. A combination of 12.5 % $^{w}/_{w}$ triacetin and 7.5 % $^{w}/_{w}$ ethanol was found to enable 5 % $^{w}/_{w}$ of fenofibrate to dissolve easily, with the additional advantage of a reduction in the viscosity of the formulations to improve ease of handling.

Any consequences from the presence of co-solvent and drug on miscibility and dispersion of the formulations were unknown, therefore ease of dispersion was assessed as per section 5.3.3. The inclusion of drug and co-solvents decreased the quality of dispersion given by formulation A1 from grade B to C. The non-lipolysing formulation containing Etocas-5 produced a dispersion classified as grade B with visible aggregates, presumably of lipophilic material evident. The non-lipolysing formulation containing Plurol oleique produced a fine emulsion with some large spherical droplets, classified as a grade C dispersion. The non-lipolysing formulation containing Plurol oleique was felt to be preferable to Etocas-5 due to lack of agglomerations in the dispersion which could restrict the availability of drug.

Miscibility status was checked by encapsulating 1 g of the final formulations into soft gelatin capsules followed by heat sealing. Three capsules were prepared for each formulation and left undisturbed at room temperature for 48 hours (the proposed period between manufacturer and *in vivo* dosage). Phase separation did not occur in any of the capsules. The lipolysing and non-lipolysing formulations developed for fenofibrate therefore met the requirements for use in the *in vivo* study. Their final formulas are listed below:

Fenofibrate Formulations

Lipolysing	% $^{w}/_{w}$	Non-lipolysing	% $^{w}/_{w}$
Soya bean oil	16	Soya bean oil	16
Crillet 4	32	Crillet 4	32
Imwitor 988	32	Plurol oleique	32
Triacetin	12.5	Triacetin	12.5
Ethanol	7.5	Ethanol	7.5

5.4.3 Development of the final formulations for cinnarizine.

The formulations developed for cinnarizine were to contain a medium chain triglyceride (FCO), for the reasons already discussed. The lipophilic surfactant of choice was oleic acid to enable demonstration of reactivation of lipolysis by a surfactant which is virtually unavailable to pancreatin as a substrate. In addition oleic acid was already known to have a high solvent capacity for cinnarizine[206] which may remove any requirements for a co-solvent.

Formulation H (Figure 5.6 h) was therefore selected as a possible lipolysing formulation with proportions of 20 % FCO, 40 % oleic acid and 40 % Crillet 4 (referred to in future discussion as formulation H1). Assessment of the lipolysing behaviour of formulation H1 by production of profiles I, II and III (results not illustrated) revealed Profile III to exhibit only 42 % of the fatty acid production shown by Profile I after one hour of digestion. As the requirement of Profile III is a minimum of 50 % reactivation of lipolysis, oleic acid could not be classified as overcoming the inhibitory effects of Crillet 4 in formulation H1.

The formulation was then manipulated in terms of the percentage of the constituents present and the surfactants used in an attempt to increase the extent to which lipolysis was reactivated. The results of this approach are shown in Table 5.4.

Composition of the formulation (%)			Percent reactivation of lipolysis
Triglyceride (FCO)	Hydrophilic surfactant	Lipophilic surfactant(s)	
20	40 (Crillet 4)	40 (oleic acid)	42
30	25 (Crillet 4)	45 (oleic acid)	44
35	20 (Crillet 4)	45 (oleic acid)	39
20	40 (Crillet 1)	40 (oleic acid)	52
30	25 (Crillet 2)	45 (oleic acid)	35
30	25 (Crillet 4)	35 (oleic acid) 10 (Imwitor 988)	60
25	25 (Crillet 4)	35 (oleic acid) 15 (Imwitor 988)	76

Table 5.4 Lipolysis shown by Profile III as a percentage of lipolysis shown by the same quantity of triglyceride in Profile I.

Table 5.4 illustrates that oleic acid within formulation H was not able to reactivate lipolysis to the percentage required (50 %) when tested in the proportions stated. Upon increasing the proportion of FCO present from 20 to 35 % in formulation H the

percentage of reactivated lipolysis remained within the same range. The accumulation of digestion products in combination with oleic acid may be responsible for inhibiting further pancreatin activity, rather than the presence of the hydrophilic surfactant.

To examine the ability of oleic acid to reactivate lipolysis in the presence of a different inhibitory hydrophilic surfactant Crillet 4, a $C_{18:1}$ 20-ethoxy sorbitan monoester used in formulation H was substituted with Crillet 1 (C_{12} 20-ethoxy sorbitan monoester) and Crillet 2 (C_{16} 20-ethoxy sorbitan monoester) in turn. Crillet 1 and 2 were selected as previous work (Figure 4.20) had already demonstrated these surfactants to be slightly less inhibitory towards pancreatin activity upon a FCO substrate than Crillet 4. The use of Crillet 1 was found to increase reactivation of lipolysis to 52 % which met the specification of Profile III, however this was still considered to be too close to the minimum requirement.

By replacing a percentage of oleic acid with Imwitor 988 the reactivation of lipolysis increased to 76 % when using proportions of 25 % FCO, 25 % Crillet 4, 35 % oleic acid and 15 % Imwitor. This combination was considered suitable for the lipolysing formulation of cinnarizine as the observed reactivation of lipolysis exceeded the 50 % requirement, whilst still demonstrating the reactivation capabilities of oleic acid. Profiles I, II and III in Figure 5.8 illustrate compliance of the formulation selected for cinnarizine with the specifications for a lipolysing formulation.

The non-lipolysing formulation of cinnarizine was to contain 25 % FCO and 25 % of Crillet 4 with the remainder of the formulation comprised of a lipophilic surfactant unable to reactivate lipolysis. Each of the following lipophilic surfactants (Etocas-5, Lauroglycol, Marlowet, Neobee and Plurol oleique) were substituted in the place of oleic acid and Imwitor 988 resulting in five potential formulations. When these were tested for miscibility as described in section 5.3.2, the formulation containing Neobee exhibited phase separation and was subsequently eliminated from the study. The lipolysing characteristics of the remaining formulations were assessed in turn by the production of a digestion profile (Profile IV). Marlowet was the only lipophilic surfactant which restricted lipolysis of the formulation to the degree shown by Profile II or below, and hence was selected for use in the non-lipolysing formulation of cinnarizine. Profile IV of the formulation containing Marlowet is illustrated in Figure 5.8 to demonstrate compliance with the requirements for a non-lipolysing formulation.

5.4.4 Dissolution of cinnarizine in the final formulations.

Dosage specifications of the *in vivo* study required the formulations of cinnarizine to contain drug at a concentration of 1.5 % $^{w}/_{w}$. Upon addition of cinnarizine to both the lipolysing and non-lipolysing formulations at the stated concentration no problems were encountered with solubility, eliminating any requirement for a co-solvent.
A final check for miscibility and ease of dispersion of the two formulations was necessary to ensure inclusion of drug had not altered these properties. The miscibility of each formulation was checked as described in section 5.4.2 and phase separation was not observed in any of the prepared capsules over a 48 hour period.
The ease of dispersion of the two formulations with drug included was determined by the method described in section 5.3.3. The lipolysing formulation gave an adequate fine emulsion with oil droplets on the surface, classified as grade C whereas the non-lipolysing formulation produced an excellent dispersion classified as grade A.
Although ideally both formulations were required to exhibit the same quality of dispersion, time constraints prevented the search for an alternative to Marlowet.
Hence the final formulations selected for cinnarizine are as listed below:

Cinnarizine Formulations

Lipolysing	% $^{w}/_{w}$	Non-lipolysing	% $^{w}/_{w}$
Fractionated coconut oil	25	Fractionated coconut oil	25
Crillet 4	25	Crillet 4	25
Oleic acid	35	Marlowet R11	50
Imwitor 988	15		

5.5 *In vitro* assessment of the formulations of fenofibrate and cinnarizine used in the *in vivo* study.

The analytical tests described in the following text were performed using the final formulations of fenofibrate and cinnarizine. The investigations were designed to examine the rate of release of drug from the formulations *in vitro* for comparison with results from the *in vivo* study. The results recorded in Tables 5.5-5.7 were determined by the analytical division of R P Scherer via HPLC within a 24 hour period from preparation of the sample.

5.5.1 The simulated gastric dispersion model.

The simulated gastric dispersion model was used to assess the rate of drug release from each formulation when the final unit dosage form was introduced into an aqueous environment. The concentration of drug dissolved in the simulated gastric fluid with time was determined. This enabled prediction of the possible proportion of drug dissolved *in vivo* upon release of the formulation into the gastric fluid in the stomach.

Method

The final formulation as used in the *in vivo* study was prepared and encapsulated into gelatin airfil capsules in quantities of 1 g. Simulated gastric fluid (100 ml) in a 500 ml round bottomed flask was slowly rotated (30 r.p.m.) by an overhead stirrer, whilst the temperature was maintained at 37°C. Two capsules of formulation were simultaneously added to the flask, which was then left to rotate for 60 minutes. Thirty and 60 minutes after commencing the experiment a 2 ml sample was removed from the flask for analysis of the concentration of drug dissolved. The experiment was repeated in duplicate for each formulation.

Results

The results from the simulated gastric dispersion model for both formulations of fenofibrate and cinnarizine are shown in Table 5.5.

	Drug in dissolved form / %			
	Fenofibrate formulations		Cinnarizine formulations	
Sample Time (minutes)	Lipolysing	Non-lipolysing	Lipolysing	Non-lipolysing
30	85, 91	24, 57	90, 93	94, 104
60	89, 101	60, 65	86, 93	98, 103

Table 5.5 Concentration of drug dissolved in the simulated gastric dispersion model as a percentage of the total drug present within the final formulations.

Results from the simulated gastric dispersions illustrate that both drugs are able to dissolve within a solution formed by simulated gastric fluid and constituents of the formulation. For all formulations a high percentage of drug dissolution occurred within 30 minutes of addition of the capsules to the fluid, the exception being the non-lipolysing formulation of fenofibrate. Results from this formulation suggest that constituents of the formulation are unable to form a solubilized phase to hold the total drug present in solution. Drug not dissolved may have precipitated in the aqueous solution or be preferentially partitioned in the lipid / surfactant droplets of the formulation.

5.5.2 The simulated intestinal digestion model.

The simulated intestinal digestion model represented digestion of the formulation within the small intestine. An increase in the concentration of drug dissolved in the simulated bile solution with time would indicate that lipolysis was releasing drug from the formulation in a state available for absorption. This model was considered more representative of the situation in the small intestine than the standard pH-stat assay due to the reduced agitation rate of the reaction mixture.

Method

Fenofibrate

Using the same apparatus as for the simulated gastric dispersion model, 100 ml of simulated bile solution (pH 7.5) with 10 µl anti-foam A was equilibrated to 37°C.

Pancreatin (250 mg), prepared as for the standard pH-stat assay, was added to the flask and allowed to distribute throughout the solution. Upon addition of 2 g of formulation to the flask, digestion commenced. A 2 ml sample was removed from the flask for analysis of the concentration of drug dissolved at 0 (before formulation addition), 30, 60, and 120 minutes. The experiment was repeated in duplicate for each formulation.

Cinnarizine

The method described for fenofibrate was adapted for cinnarizine as the solubility of this drug was known to vary with pH. The experiment was performed as described above and the digestion left to proceed for the required period of time (30, 60 or 120 minutes) at which point the reaction mixture was transferred to the pH-stat and back-titrated to pH 7.5. A 2 ml sample was immediately removed for analysis of the concentration of drug dissolved. This method therefore involved a separate digestion experiment to obtain each sample. The whole experiment was repeated in duplicate for each formulation.

Results

Results from the simulated intestinal digestion model for both formulations of fenofibrate and cinnarizine are shown in Table 5.6.

	Drug in dissolved form / %			
	Fenofibrate formulations		Cinnarizine formulations	
Sample Time (minutes)	Lipolysing	Non-lipolysing	Lipolysing	Non-lipolysing
30	83, 88	29, 64	ND	97
60	91, 93	37, 51	ND	97
120	90, 91	39, 52	ND	96, 100

Table 5.6 Concentration of drug dissolved in the simulated intestinal digestion model as a percentage of the total drug present within the final formulations. (ND = Not determined).

Results from the simulated intestinal digestion model reflect the solubility of drug at various time intervals throughout a slow digestion. The same pattern was seen as for the gastric dispersion experiments, with high levels of drug solubility from the 30

minute sample onwards except from the non-lipolysing formulation of fenofibrate. The lipolysing formulation of cinnarizine could not be analysed as the viscosity of the sample prevented analysis by HPLC.

5.5.3 Effect of digestion mixture and filtration upon the assay of dissolved drug.

The sample solutions from the simulated gastric dispersion and intestinal digestion models were complex mixtures of drug, the formulation, components from the simulated gastric fluid or bile solution and other possible constituents in the reaction mixture.

To ensure that all of the drug dissolved within the complex sample solution was available for analysis via the HPLC method used, a control experiment was devised. The control experiment involved digestion of the final formulation under conditions of the standard pH-stat assay with drug omitted. A known concentration of dissolved drug was added to two samples of the resulting digestion reaction medium. One of these samples was filtered before analysis by HPLC whilst the other was not.

This approach enabled examination of the availability of drug for assay by HPLC when present within the complex digestion mixture. In addition an assessment was also possible to determine if the process of filtration, necessary for the HPLC assay, reduced the concentration of drug detected through drug binding to the filter (despite the standard precaution taken of discarding the first 0.5 ml of filtrate).

Method

Each formulation was digested under conditions of the standard pH-stat assay using 2 g of the final formulation with drug omitted in each case. The assay was performed at a pH of 7.5 for a period of two hours. Upon completion of the assay two 1 ml samples of reaction mixture were removed. One ml of standard drug solution (fenofibrate 0.1 % $^{w}/_{v}$ or cinnarizine 0.03 % $^{w}/_{v}$ as appropriate) was then added to each sample. One of the samples was filtered through a 0.2 μm filter (discarding the first 0.5 ml of filtrate) before HPLC analysis to determine the concentration of drug dissolved whereas the other was not.

Results

Results from the above experiment are shown in Table 5.7 for the four formulations.

	Fenofibrate formulations		Cinnarizine formulations	
Sample	Lipolysing	Non-lipolysing	Lipolysing	Non-lipolysing
Unfiltered	98, 100	97, 100	106	95
Filtered	94	94	100	108

Table 5.7 Percentage of standard drug solution available for HPLC analysis when combined with digestion components pre and post filtration.

The results shown in Table 5.7 suggest that the majority of drug present is available for assay when in the presence of components created by digestion of the final lipid-based formulations. It could not be stated that filtration decreased the concentration of drug determined when compared to the corresponding unfiltered sample. This suggests that any alteration in results obtained via HPLC due to drug binding to the filter was insignificant when the precaution of discarding the initial filtrate was taken.

5.5.4 Conclusions of *in vitro* assessment of the final formulations.

The high levels of drug solubility determined in the simulated gastric dispersion model suggests digestion of these formulations is not required to release the drug in a dissolved form into the surrounding aqueous media. It could be proposed that the fine dispersions promote drug diffusion from the lipid droplets and the solubilization phase formed by the constituents of the formulation is able to hold drug in solution.
The non-lipolysing formulation of fenofibrate appears in some manner to prevent drug dissolution in aqueous fluid. It seems possible that drug is preferentially partitioned into droplets formed from the formulation. The observation of droplets at the surface and bottom of the flask during assessment of the dispersion formed by the non-lipolysing formulation of fenofibrate gives some support to this supposition.
Additional evidence for preferential partitioning of fenofibrate into droplets of the lipid-based formulation is provided by the results in Table 5.7. The high solubility of fenofibrate when added in a dissolved form to the digested formulation (Table 5.7) suggests that drug dissolution is only impeded if drug is already within the formulation.

As the overall intended purpose of this investigation was to demonstrate the advantage of a lipolysing versus a non-lipolysing formulation the results from these *in vitro* evaluations were not promising. The lipolysing formulations should have demonstrated minimal drug dissolution within the gastric fluid with the majority of drug remaining partitioned in the lipid formulation. Upon digestion the concentration of drug dissolved in the reaction mixture should have increased with time representing the advantage of digestion to release drug previously partitioned in the lipid phase. Drug in the non-lipolysing formulations would ideally dissolve to the same extent in the gastric dispersion as seen from the lipolysing system with no subsequent increase in the concentration of drug dissolved in the digestion model.
As far as could be determined the non-lipolysing formulation of fenofibrate was the only formulation behaving as intended *in vitro* with a significant proportion of drug remaining partitioned in the formulation unavailable for release by lipolysis.

5.6 Comparative pharmacokinetics of the fenofibrate and cinnarizine lipid-based formulations from the *in vivo* study.

The *in vivo* study described below was commissioned by R P Scherer and carried out in entirety by Corning Hazleton[207]. An outline of the study and results have been included within this chapter to assess the influence of the lipolysing and non-lipolysing lipid-based formulations (developed in section 5.4) upon drug bioavailability.
The overall intention of the study was to provide support for formulation of hydrophobic drugs in digestible lipid systems, by demonstration of improved bioavailability compared to that obtained from conventional oral dosage forms.
The study was thus designed to compare the pharmacokinetics of cinnarizine and fenofibrate from three formulations containing the same dose of drug. The formulations to be examined were the current marketed product and the lipolysing and non-lipolysing lipid-based formulations developed in section 5.4.
To enable quantification of drug bioavailability the pharmacokinetic parameters of maximum plasma concentration (C_{max}), the time of maximum concentration (t_{max}), and the area under the curve (AUC_{0-24}) were used. C_{max} and t_{max} were determined by visual

inspection of the pharmacokinetic profiles whereas AUC_{0-24} was quantified computationally.

The study design used was a randomised three way balanced cross-over in six male beagle dogs, with three dosing intervals separated by a 14 day washout period. The dogs were an appropriate model as capsules could be administered in the same manner as would be done for humans and the drug plasma levels were quantifiable via HPLC analytical methods.

Method

Fenofibrate

The three formulations examined in the study are listed below:

Formulation A One capsule (hard gelatin capsule shell) containing 100 mg of fenofibrate in powder form (Lipantil®).

Formulation B Two capsules each containing 50 mg of fenofibrate in 1 g of the lipolysing formulation.

Formulation C Two capsules each containing 50 mg of fenofibrate in 1 g of the non-lipolysing formulation.

After each dosing 11 plasma samples were taken from the dogs over a 24 hour period and the concentration of fenofibrate in the plasma was assayed via HPLC with ultraviolet detection.

Cinnarizine

The three formulations examined in the study are listed below:

Formulation A Two tablets each containing 15 mg of cinnarizine (Stugeron®).

Formulation B Two capsules each containing 15 mg of cinnarizine in 1 g of the lipolysing formulation.

Formulation C Two capsules each containing 15 mg of cinnarizine in 1 g of the non-lipolysing formulation.

After each dosing 11 plasma samples were taken from the dogs over a 24 hour period and the concentration of cinnarizine in the plasma was assayed via HPLC using a mass spectrometry detection method.

Results

Fenofibrate

The mean plasma concentrations of fenofibrate determined for each formulation are illustrated in Figure 5.9 (up to 10 hours post-dose) and Table 5.8 summarizes the mean pharmacokinetic parameters obtained from the study.

Parameter		Formulation A Mean value	Formulation B Mean value	Formulation C Mean value *
C_{max}	(μg / ml)	11.03	5.64	5.38
t_{max}	(h)	1.3	1.7	1.8
AUC_{0-24}	(μg.h / ml)	34.14	34.94	28.99

Table 5.8 Mean pharmacokinetic parameters from the fenofibrate *in vivo* study. (reproduced from reference[110]). * n = 5 data sets

Corning Hazleton concluded that all formulations demonstrated similar t_{max} values (Table 5.8). Formulation A had a C_{max} value twice that of either formulation B or C. However this was not reflected in the comparative bioavailability of the formulations (from the AUC_{0-24} values), which was similar for all three formulations (Table 5.8).

Cinnarizine

The mean plasma concentrations of cinnarizine determined for each formulation are illustrated in Figure 5.10 (up to 10 hours post-dose) and Table 5.9 summarizes the mean pharmacokinetic parameters obtained from the study.

Parameter		Formulation A Mean value *	Formulation B Mean value	Formulation C Mean value
C_{max}	(ng / ml)	94.49	165.36	100.02
t_{max}	(h)	1.3	1.6	1.8
AUC_{0-24}	(ng.h / ml)	406.07	667.73	451.51

Table 5.9 Mean pharmacokinetic parameters from the cinnarizine *in vivo* study. (reproduced from reference[111]). * n = 4 data sets.

Corning Hazleton summarized the results from the cinnarizine study as follows; all three formulations had similar times to C_{max} (Table 5.9). Formulation B had a C_{max} value which was 60 % greater than that shown by formulations A and C (Table 5.9).

The C_{max} values were also reflected by the AUC_{0-24} (Table 5.9). Therefore formulation B was stated to have the highest maximum plasma concentration and bioavailability of the three formulations tested, based on AUC values from 0 to 24 hours.

5.6.1 Discussion of results from the *in vivo* study.

Fenofibrate

The mean plasma concentrations of fenofibrate (Figure 5.9) showed no difference in bioavailability between the three formulations tested, with the highest C_{max} value recorded from the proprietary capsule. The *in vitro* assessment of the same lipid-based formulations of fenofibrate had demonstrated poor dissolution of fenofibrate upon release of the non-lipolysing system into gastric fluid, when compared to results from the lipolysing formulation (Table 5.5). This was proposed to be due to drug remaining partitioned within droplets of the formulation. The difference in dissolution behaviour between the two lipid-based formulations was not reflected by the results from the *in vivo* study.

As the same bioavailability was achieved from the two lipid-based formulations *in vivo* this suggests the non-lipolysing characteristics of formulation C may have been overcome by physiological mechanisms, thus allowing digestion to release drug partitioned in the lipid droplets. Alternatively bioavailability from both formulations may have been influenced by an unknown mechanism. The lipid-based formulations of fenofibrate contained a high concentration of co-solvents, which are known to undergo rapid dilution upon release into aqueous fluid[188]. This could result in precipitation of the drug, and thus a restriction to bioavailability from both formulations by addition of a drug dissolution stage. The *in vitro* assessments do not support this proposed mechanism as no restriction to drug solubility was noted for the lipolysing formulation in gastric fluid (Table 5.5), although this could possibly be due to the fact that the co-solvents were still present within the closed system.

The proportion of hydrophilic components within the formulations of fenofibrate was high, with 52 % of the formulation comprised of hydrophilic surfactant and co-solvents compared to 25 % for the cinnarizine systems. High concentrations of hydrophilic surfactant have been suggested to result in the solubilization and

entrapment of poorly water-soluble drugs within the micelles thus formed,[192] reducing levels of drug absorption. In any event it is clear that formulation within a lipolysing system was in the case of fenofibrate ineffective in terms of improving drug bioavailability over that from a formulation which exhibited non-lipolysing behaviour *in vitro*.

Cinnarizine

In the case of cinnarizine the mean plasma concentration of the lipolysing formulation was raised compared to the non-lipolysing formulation and the proprietary tablet (Figure 5.10). This difference could not be accounted for by the *in vitro* model of gastric dispersion, which demonstrated both cinnarizine lipid-based formulations to be equally effective with regards to holding the majority of drug present in a dissolved state in aqueous fluid (Table 5.5).

I*n vitro* tests also suggested lipid digestion was not required to release cinnarizine from the non-lipolysing system (Table 5.5), therefore the reduced bioavailability from this formulation may possibly relate to differences between the constituents of the formulations. The oleic acid and Imwitor 988 surfactant within the lipolysing formulation are or contain natural digestion products. These may promote the formation of intestinal mixed micelles upon entry into the intestine, which subsequently solubilize drug and aid transport to the microvilli for absorption. The non-lipolysing formulation contains no such products and may initially release drug in a dissolved state as a result of the excellent dispersion behaviour shown by this formulation *in vitro* (section 5.4.4). If drug is not absorbed rapidly, upon further dilution of the solubilized phase with GI fluid, precipitation of the drug may be expected thus accounting for the reduced bioavailability.

The benefit to drug bioavailability conferred by lipid-based formulations able to form a fine dispersion upon release into gastric fluid has been demonstrated by a similar *in vivo* study designed by R P Scherer[208]. Using the same study design as for fenofibrate and cinnarizine above, two lipid-based formulations were compared with a proprietary solid oily suspension, with each formulation containing 100 mg of progesterone. The lipid formulations were SEDDS which were able to undergo lipolysis within the

standard pH-stat assay. Figure 5.11 illustrates the mean plasma profile from each formulation. The benefit to bioavailability of the lipolysing SEDDS was evident from the 8 to 10 fold increase in C_{max} and 5 to 7 fold increase in AUC_{0-24} compared to the proprietary oral solid dosage form.

5.7 Conclusions

Care needs to be exercised when utilizing lipophilic and hydrophilic surfactants to improve the dispersion of a lipid-based formulation. Inclusion of certain hydrophilic surfactants may prevent lipolysis of a lipid-based formulation. However the use of a lipophilic surfactant in the same formulation may be able to overcome the inhibitory effect of the hydrophilic surfactant upon lipolytic enzymes. Only certain lipophilic surfactants have the ability to do this, with effective reactivation of lipolysis appearing to relate to the production of lipolytic products, which may in turn promote colipase partitioning at the interface. In any event lipophilic surfactants containing partial digestion products as part of their composition are a good first choice for a formulation. Not only have these proved highly successful in this work at reactivating lipolysis but they are also devoid of toxicity problems if one based solely upon natural triglyceride digestion products is selected.

The formulation exercise described within this chapter highlights the complexity of formulating a lipid-based system and tends to suggest that for the near future development of a universal lipid-based formulation for hydrophobic drugs is remote. Careful selection of constituents for the formulation to maximize drug solubility within the system and hopefully to avoid the use of co-solvents (which may lead to subsequent precipitation of drug) is essential and peculiar to each drug.

The issue of bioavailability enhancement through administration of drug in a lipolysing as opposed to a non-lipolysing system (when assessed *in vitro*) remains unclear. Results from the *in vivo* study discussed in this chapter are difficult to interpret due to variations between the formulations such as quality of dispersion and the use of co-solvents. *In vitro* assessments of the formulations did not relate directly to the *in vivo* bioavailability data obtained. Although improved bioavailability from a lipid-based formulation able to undergo lipolysis is unproven, it may be prudent to develop lipid-

based formulations which display *in vitro* lipolysing behaviour until further knowledge is gained.

Figure 5.1a Digestion profiles of lipophilic surfactants (1 g) which displayed a digestion rate below the simulated bile blank profile for part of the assay period.

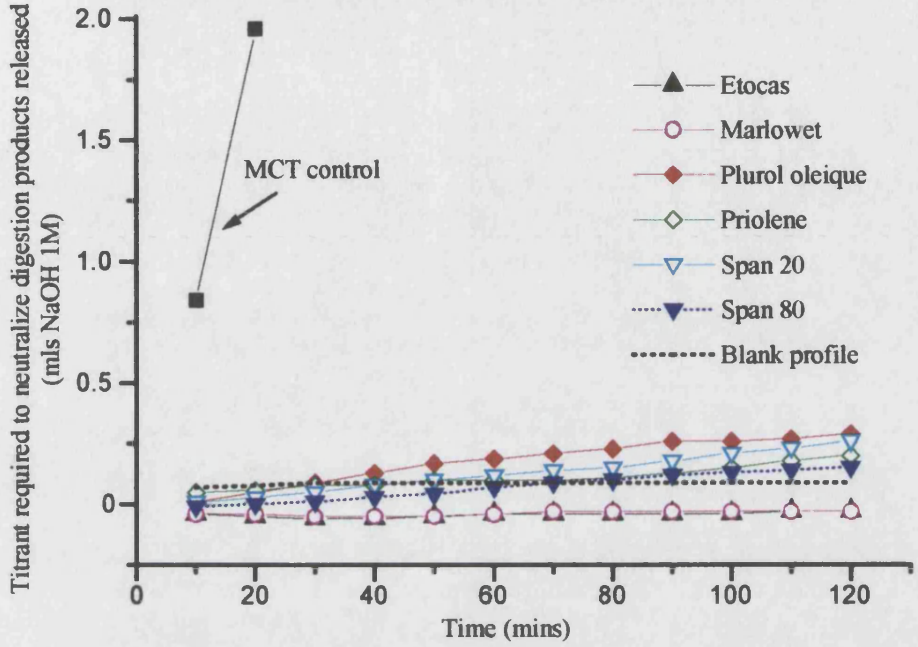


Figure 5.1b Digestion profiles of lipophilic surfactants (1 g) for which the extent of digestion exceeded the simulated bile blank profile for the total duration of the assay.

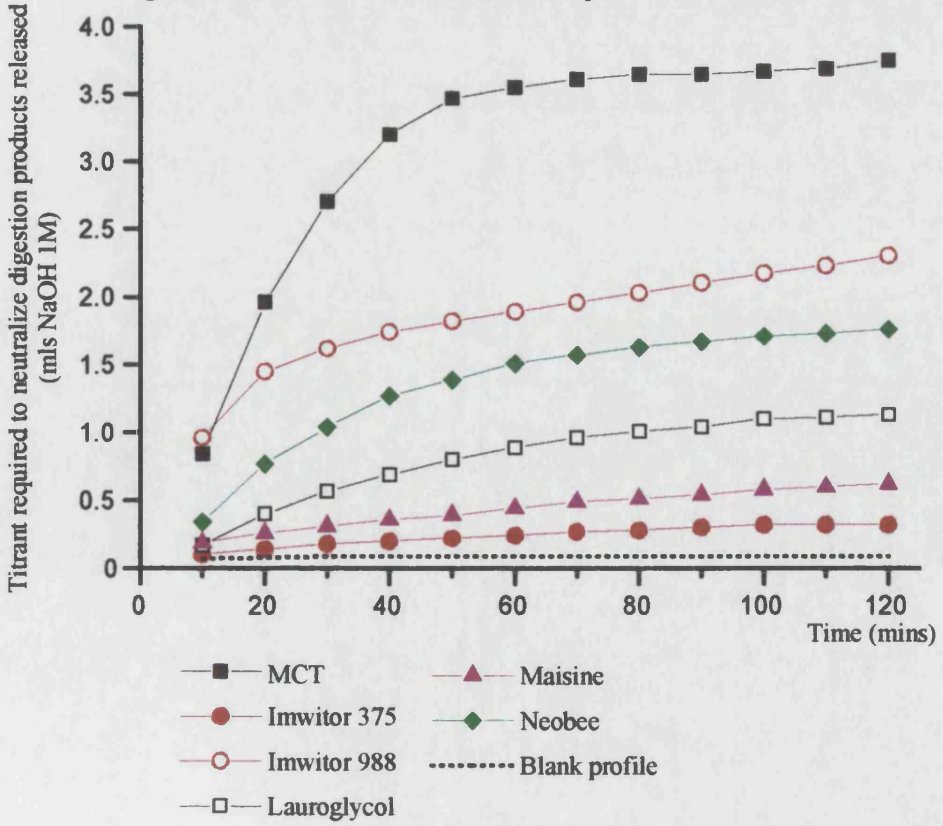


Figure 5.2 Ability of lipophilic surfactants to reactivate the non-lipolysing model of lipolysis.

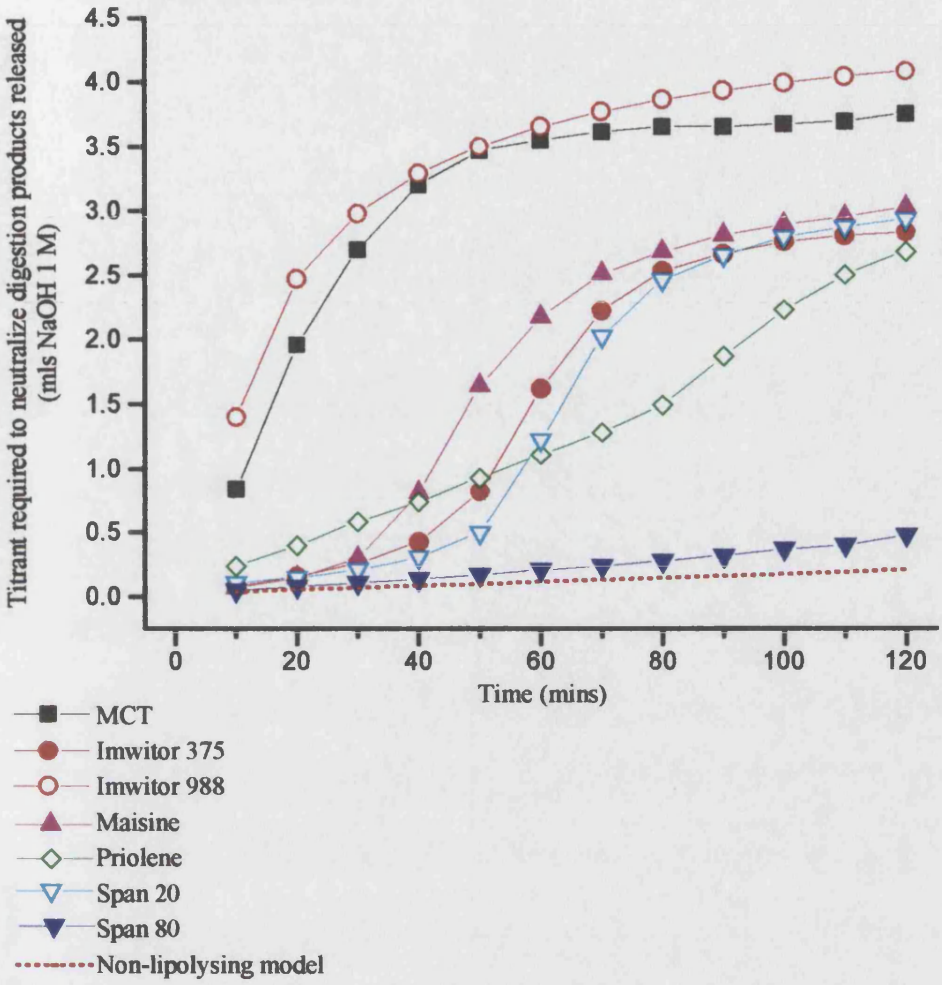


Figure 5.3 Digestion profiles to illustrate the variation in liberation of acidic components when various constituents of the non-lipolysing model (and Imwitor 988) were present.

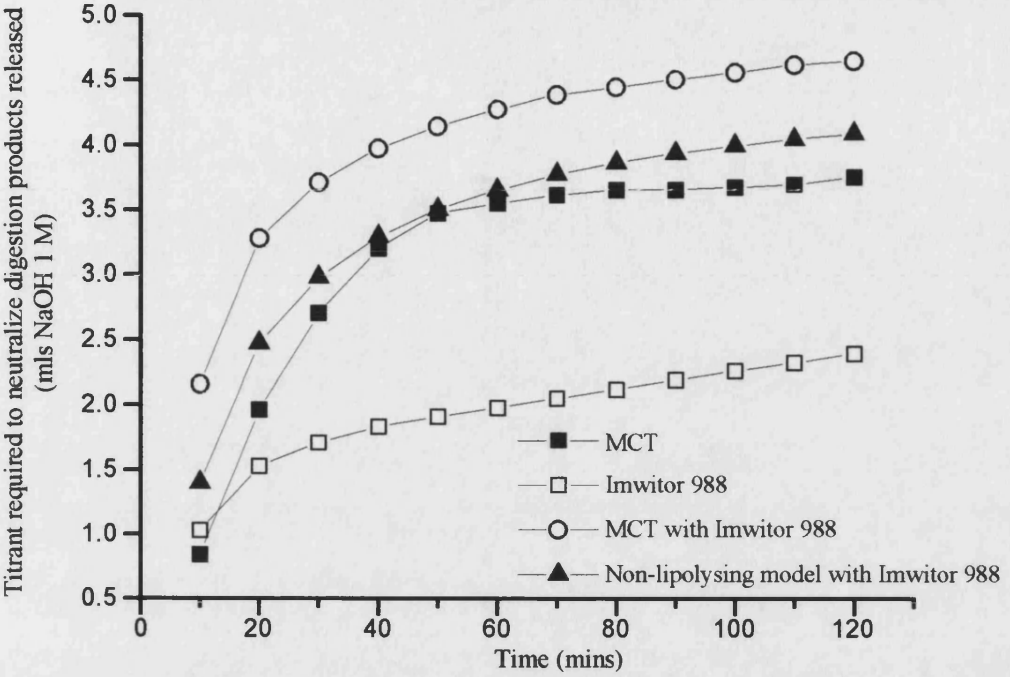


Figure 5.4 Digestion profiles to illustrate the variation in liberation of acidic components when various constituents of the non-lipolysing model (and Imwitor 375) were present.

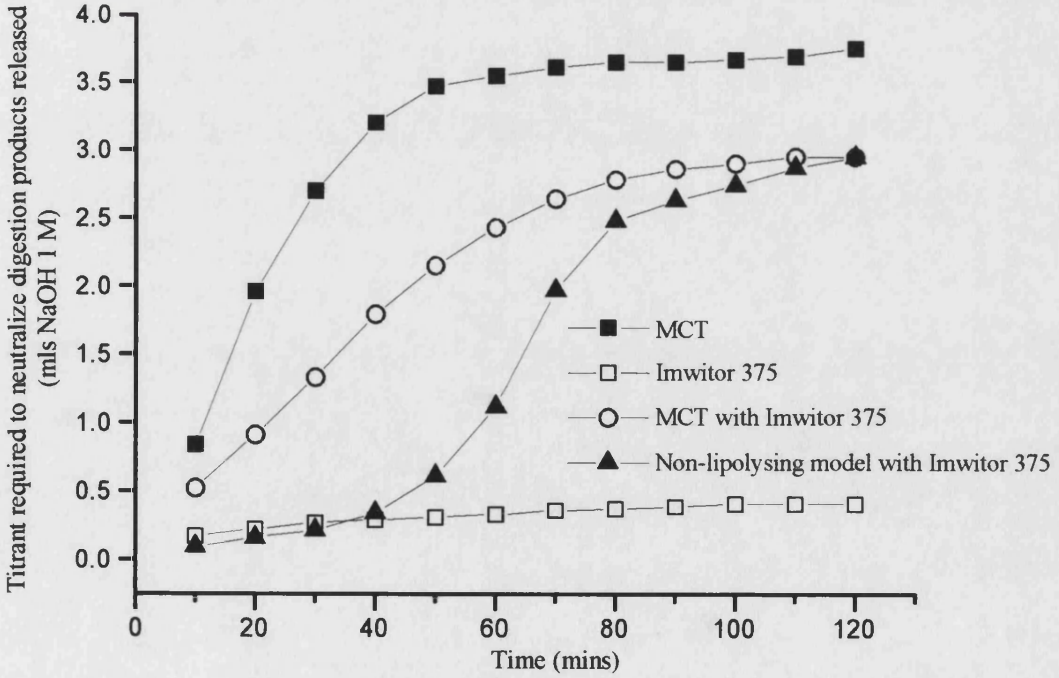


Figure 5.5a Solubility of Fenofibrate determined in specified micellar solutions. (standard deviation from 3 replicates).

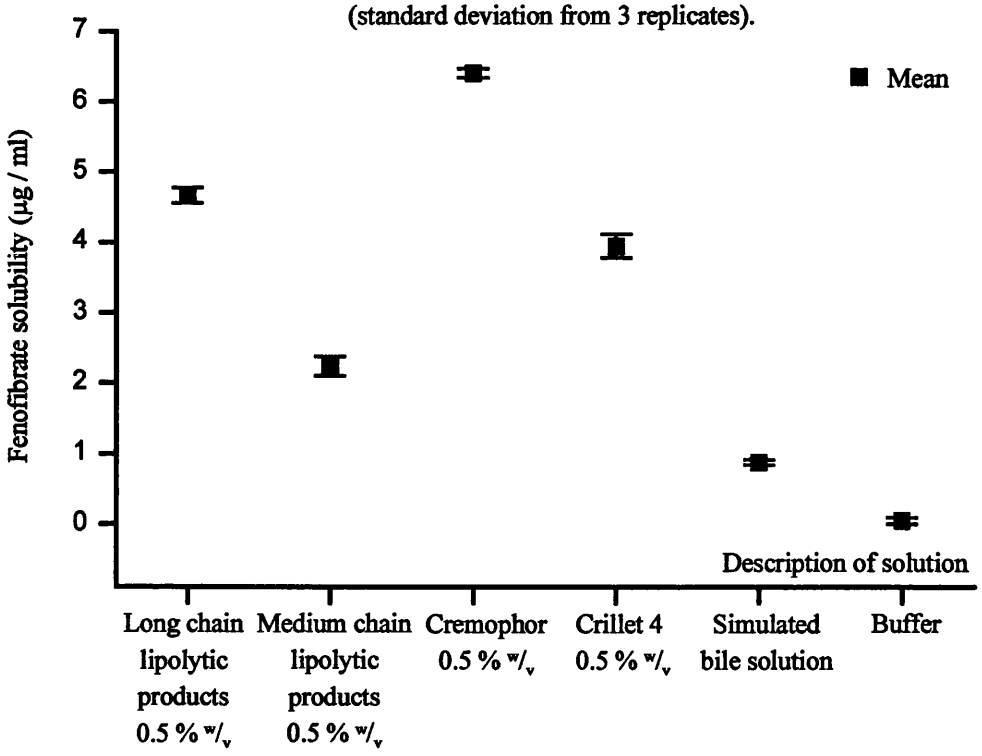
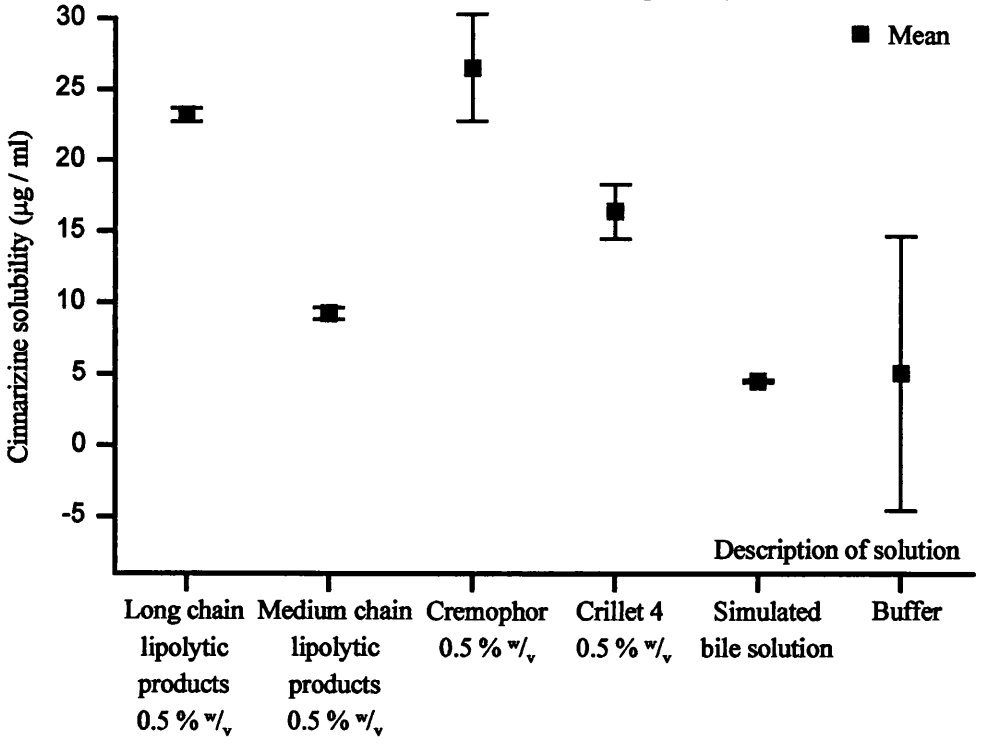


Figure 5.5b Solubility of Cinnarizine determined in specified micellar solutions. (standard deviation from 3 replicates).



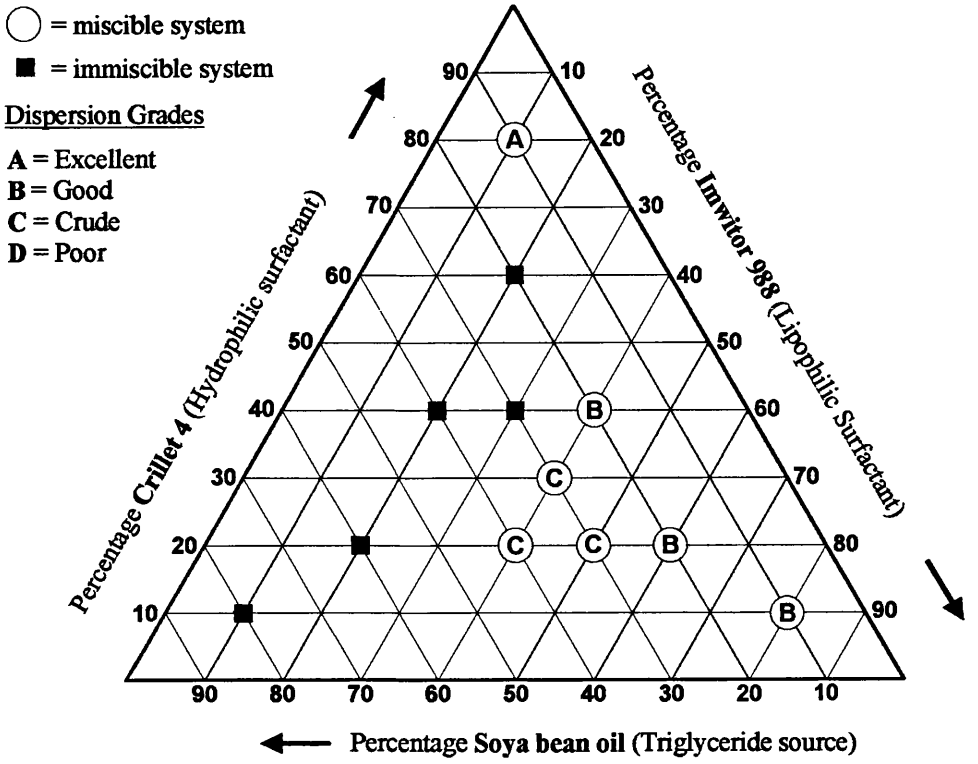


Figure 5.6 (a) Ternary Phase Diagram for **Formulation A** displaying miscibility and score for dispersion for systems of various composition.

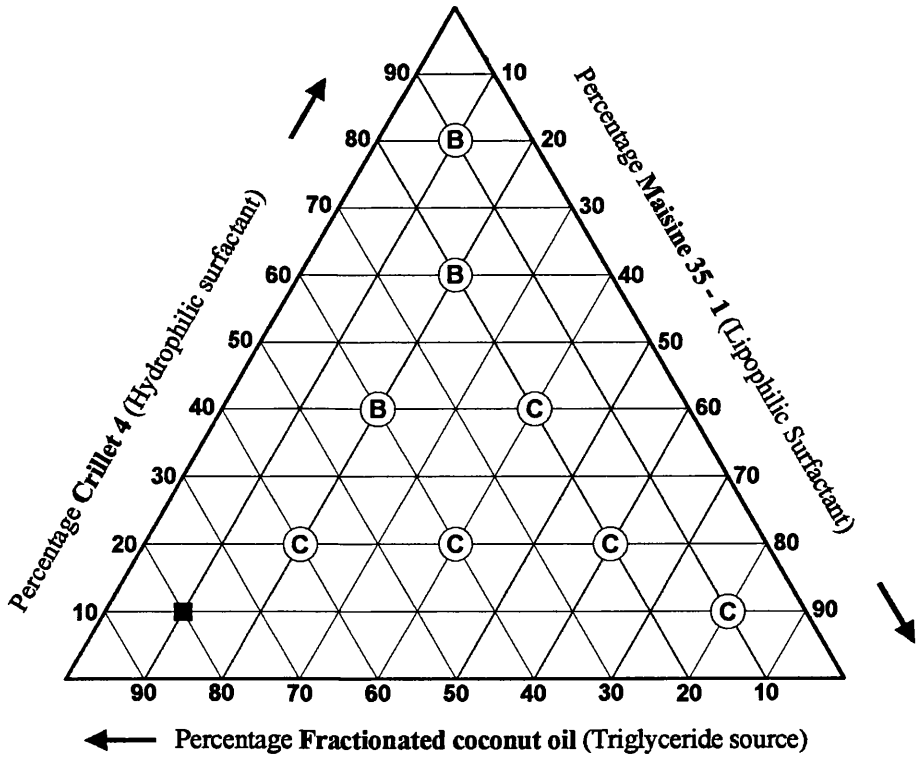


Figure 5.6 (b) Ternary Phase Diagram for **Formulation B** displaying miscibility and score for dispersion for systems of various composition.

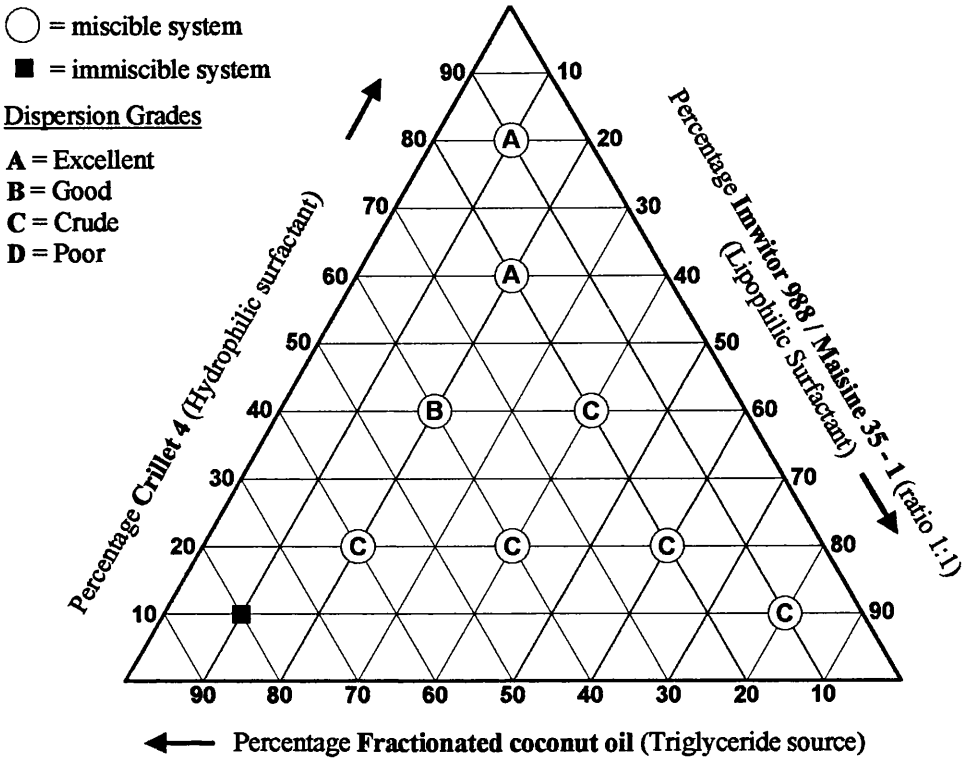


Figure 5.6 (c) Ternary Phase Diagram for **Formulation C** displaying miscibility and score for dispersion for systems of various composition.

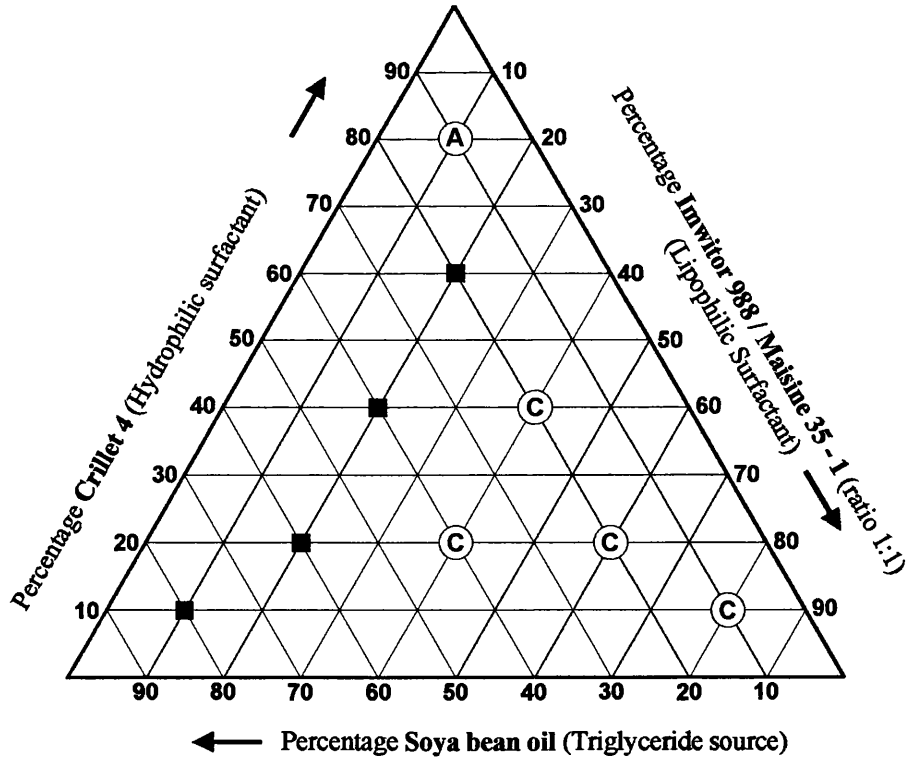


Figure 5.6 (d) Ternary Phase Diagram for **Formulation D** displaying miscibility and score for dispersion for systems of various composition.

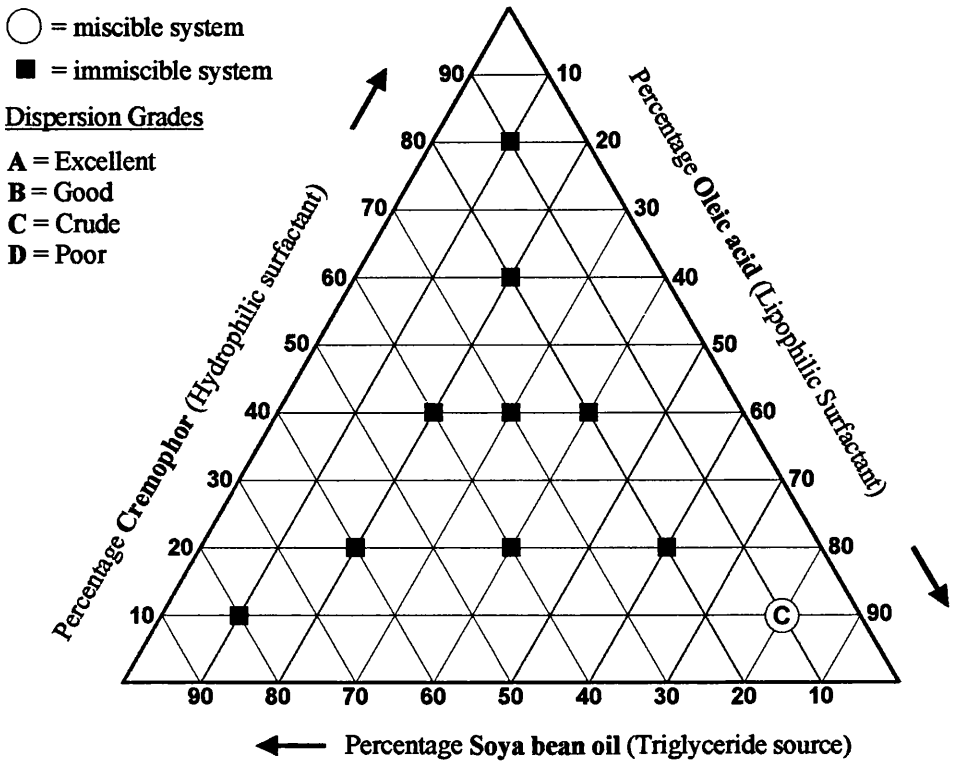


Figure 5.6 (e) Ternary Phase Diagram for **Formulation E** displaying miscibility and score for dispersion for systems of various composition.

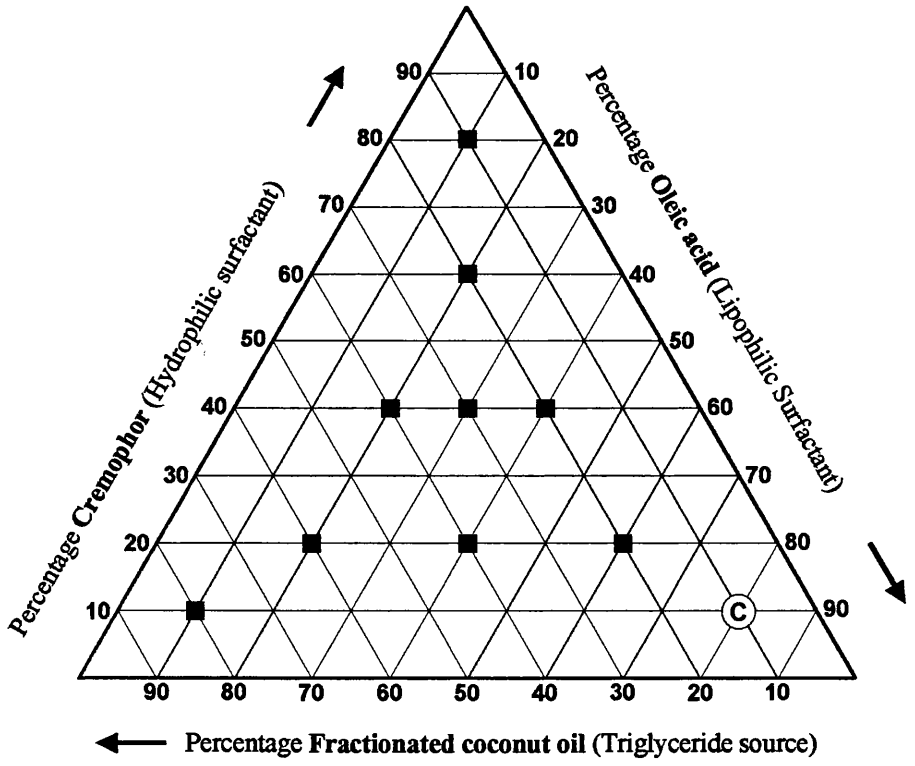


Figure 5.6 (f) Ternary Phase Diagram for **Formulation F** displaying miscibility and score for dispersion for systems of various composition.

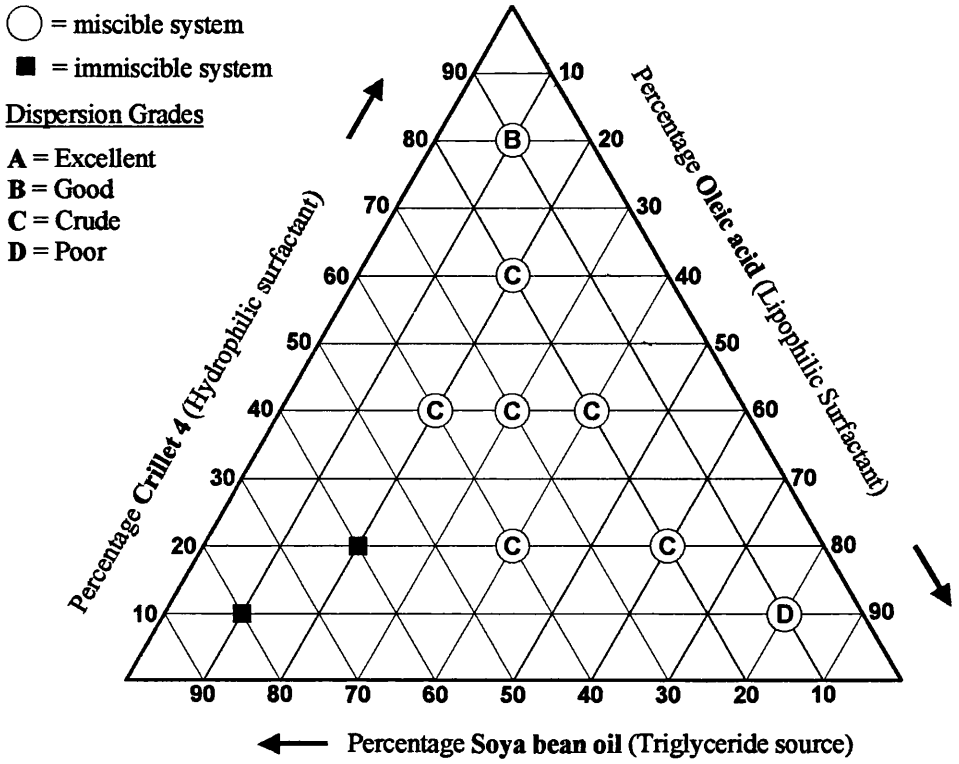


Figure 5.6 (g) Ternary Phase Diagram for **Formulation G** displaying miscibility and score for dispersion for systems of various composition.

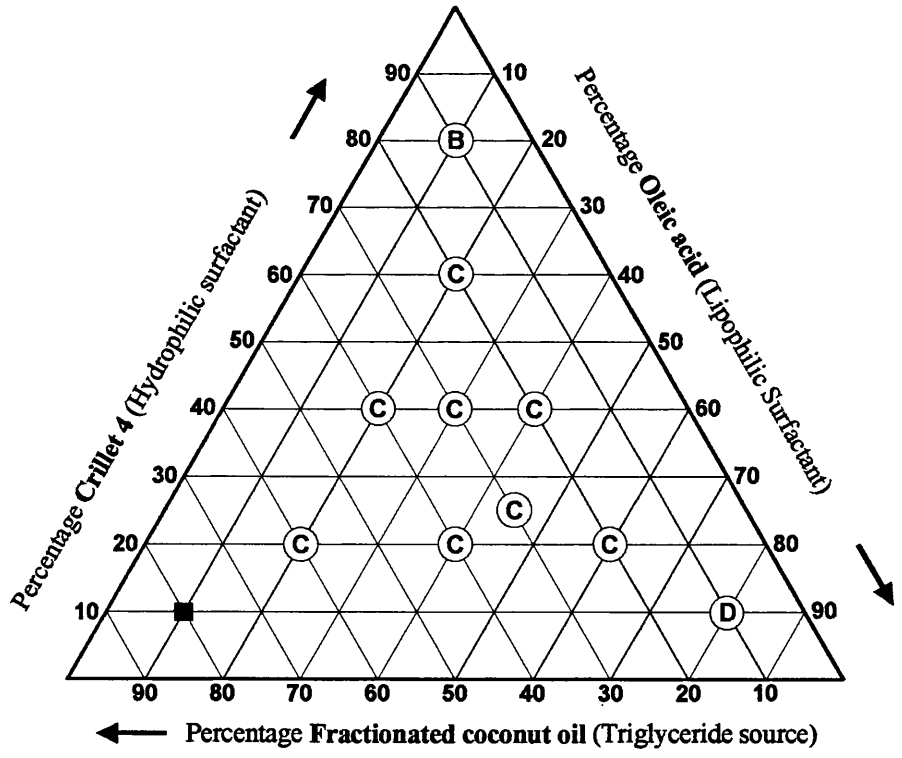


Figure 5.6 (h) Ternary Phase Diagram for **Formulation H** displaying miscibility and score for dispersion for systems of various composition.

Figure 5.7 Digestion profiles to demonstrate compliance of the Fenofibrate formulations with the lipolysing criteria specified by Profiles I to IV.

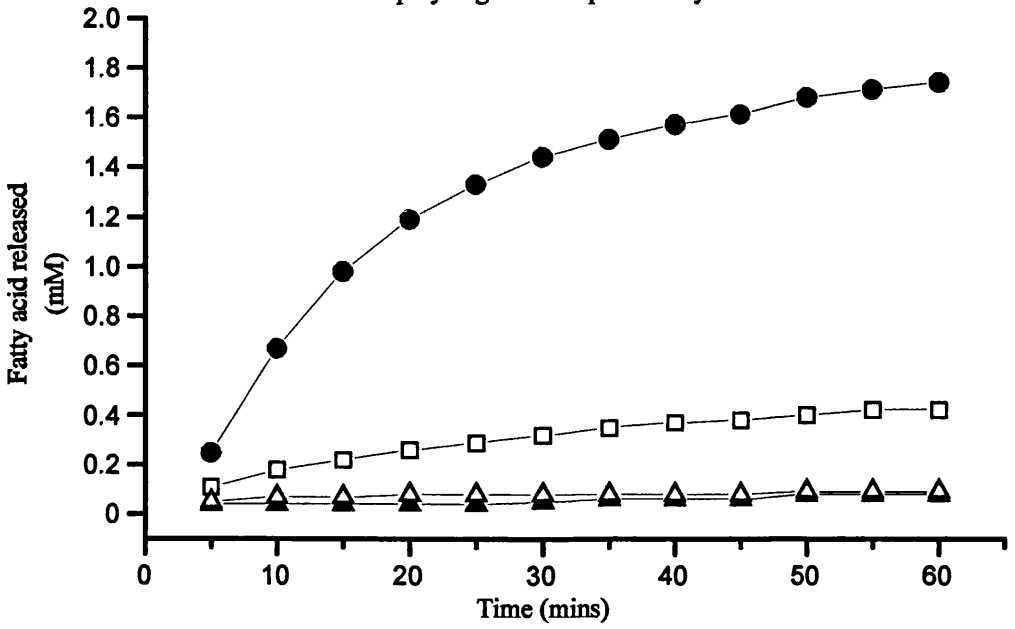
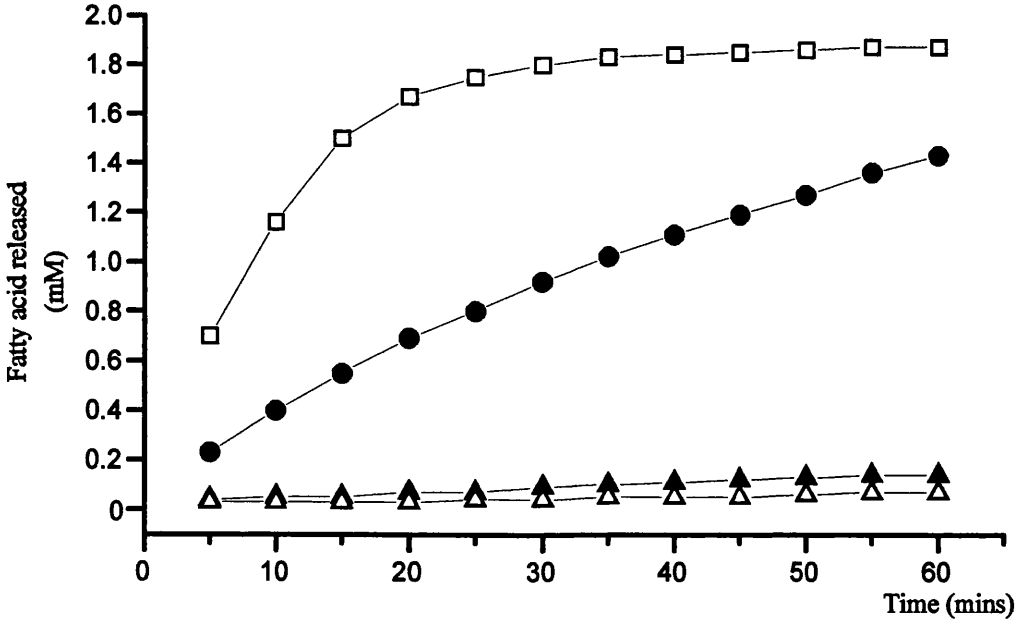


Figure 5.8 Digestion profiles to demonstrate compliance of the Cinnarizine formulations with the lipolysing criteria specified by Profiles I to IV.



Legend for Figures 5.7 & 5.8

—□— Profile I Triglyceride

—▲— Profile II Triglyceride + hydrophilic surfactant

—●— Profile III Lipolysing formulation

—△— Profile IV Non-lipolysing formulation

Figure 5.9 The mean plasma concentrations of Fenofibrate (μg / ml) determined for each formulation. (Reproduced from reference[110]).

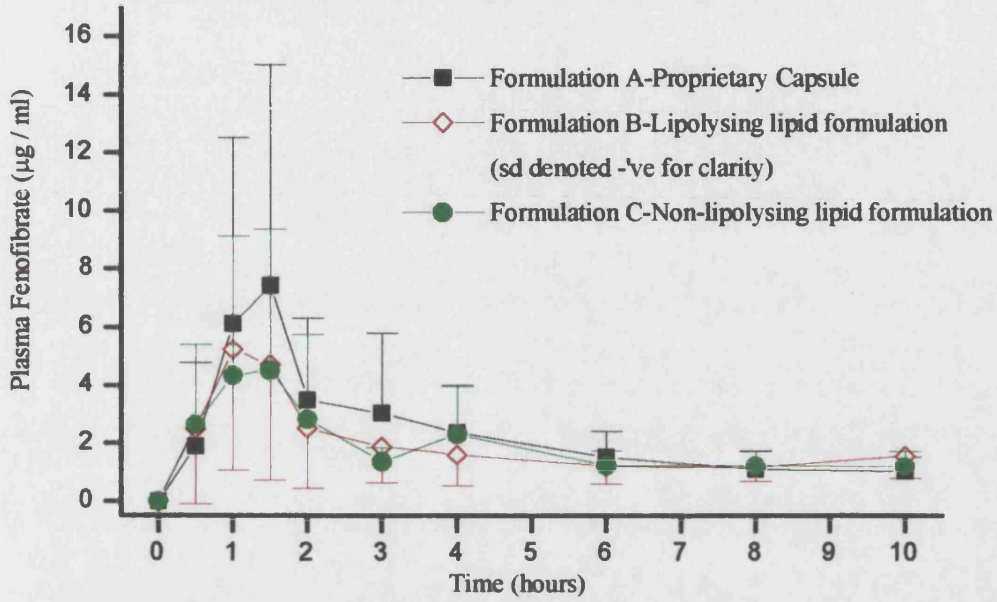


Figure 5.10 The mean plasma concentrations of Cinnarizine (ng/ml) determined for each formulation. (Reproduced from reference[111]).

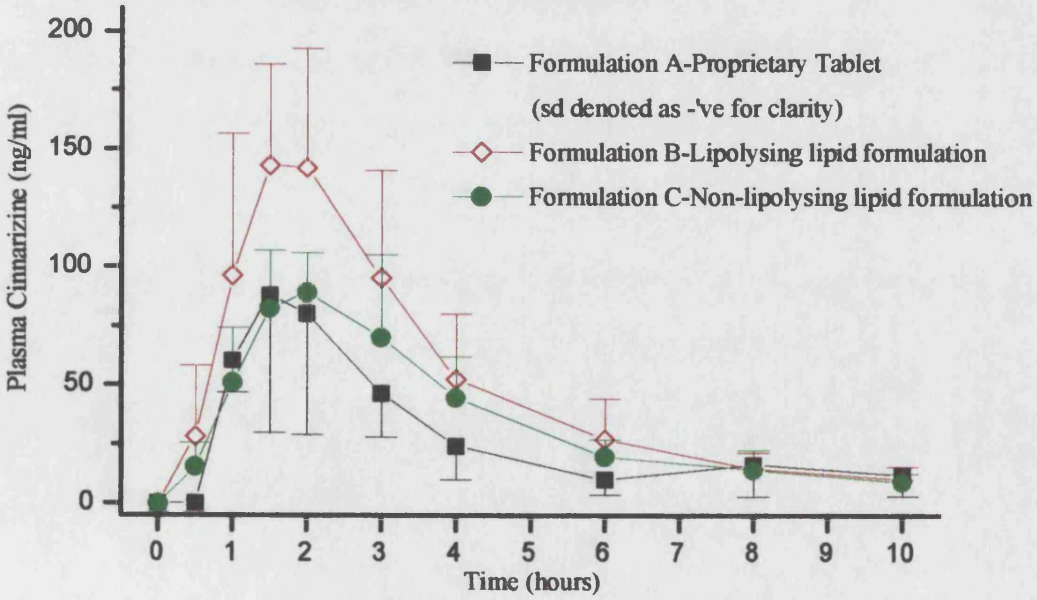
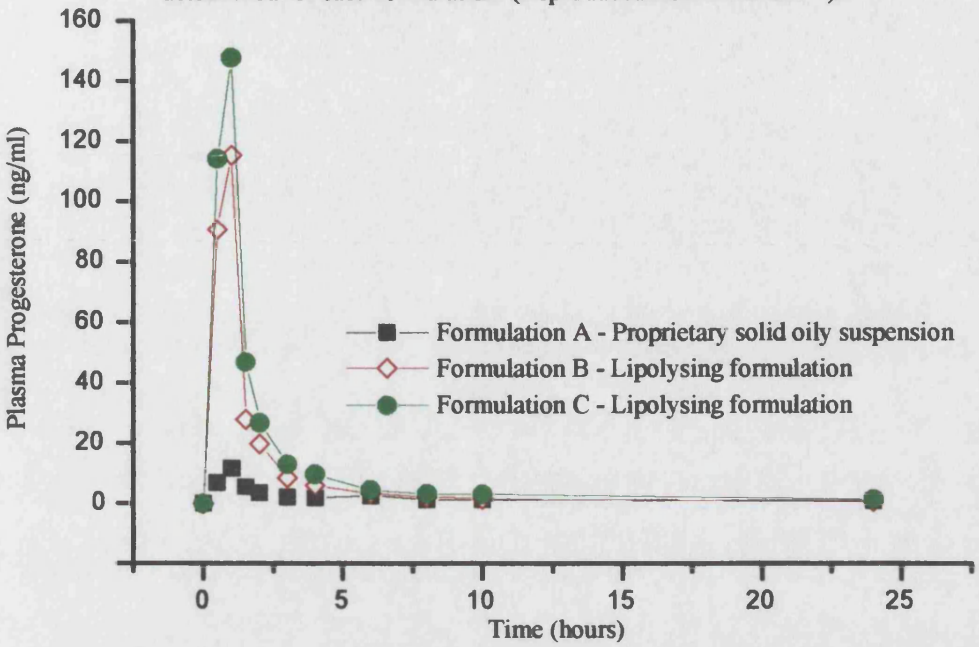


Figure 5.11 The mean plasma concentrations of Progesterone (ng/ml) determined for each formulation. (Reproduced from reference[208]).



Chapter 6 - Solubility enhancement of steroidal compounds in micellar solutions of physiological surfactants.

6.1 Introduction

The bioavailability of drugs with poor aqueous solubility has in some cases been notably improved upon administration with or after food[4]. This infers that processes stimulated during the digestion of food may in some way enhance drug levels in the blood. Hydrophobic drugs are normally readily absorbed across the GI membrane however their slow dissolution in aqueous GI fluid may be the rate limiting step with regards to bioavailability.

A major change which occurs in the duodenum upon ingestion of food is the release of biliary secretions containing bile salts and phospholipids (predominantly lecithin[8]), in high concentrations with cholesterol. Both bile salts and lecithin possess surfactant properties which may enhance the dissolution rate of hydrophobic drugs via the possible mechanisms of wetting and solubilization.

The presence of physiological surfactants in the GI fluid could increase the wettability of the surface of a drug with a resultant increase in the dissolution rate. The wettability of a solid is expressed by the contact angle between a solid surface and a liquid[165]. A contact angle close to zero will result in liquid spontaneously wetting the solid, whereas values of 90° or greater prevent liquid penetration into the capillary pores of a powder and tend to inhibit wetting[209]. Some hydrophobic drugs agglomerate upon fine particle size reduction. In this case the shape of the resultant powder aggregate and pore size, in addition to contact angle, will influence the ability of liquid to penetrate the powder capillary and wet the solid surface[210]. Physiological surfactants may increase the dissolution rate by reducing the interfacial tension between the surface of the drug and GI fluid. The contact angle will thus be decreased, increasing wettability of the drug and the effective surface area of the drug available for dissolution[104].

An increase in the aqueous solubility of a drug may also increase the dissolution rate. Although solubility of a drug is an intrinsic characteristic it can be enhanced by a mechanism known as solubilization. This has been defined as 'a particular mode of bringing into solution substances that are otherwise insoluble in a given medium,

involving the previous presence of a colloidal solution whose particles take up and incorporate within or upon themselves the otherwise insoluble material'[211]. The monomers of surfactants (when present above the CMC) tend to aggregate together to form micelles, which are capable of rapid breakdown and reformation. Within the micellar structures the insoluble material becomes incorporated and is said to be solubilized.

From the 1960's literature reports of the ability of bile salts to improve drug solubility, dissolution rate and absorption have been published. *In vitro* experiments by Bates et al demonstrated an increase in the dissolution rate[212] and solubility[213] of the poorly water-soluble drugs griseofulvin and hexoestrol when in the presence of bile salts. They suggested this effect was due to solubilization of drug within bile salt micelles. *In vivo* results from Meli et al[214], using comparisons between bile duct-cannulated rats and control animals, revealed the presence of endogenous bile to enhance the absorption rate of ethynylestradiol 3-cyclopentyl ether from the small intestine. Absorption of the poorly water-soluble drug, sulfadiazine was compared using rat *in situ* intestinal loops under various conditions of bile flow[215]. An increase in absorption was observed when bile flow was enhanced. The view that this was probably due to increased drug solubility via micellar solubilization was supported by a concurrent *in vitro* solubility study.

In an attempt to discern the mechanism by which bile salt can enhance drug dissolution rate, Miyazaki et al[216] examined the effect of bile salt on the *in vitro* dissolution of indomethacin and phenylbutazone. Results indicated that the enhanced dissolution of the two drugs, both of which have poor aqueous solubility, was mediated by two different effects related to the presence of bile salts. Enhanced dissolution of indomethacin was suggested to be due to increased solubility via micellar solubilization, whereas for phenylbutazone wetting effects may have been responsible by increasing the effective surface area of the drug.

Bakatselou et al[217] also carried out an *in vitro* investigation to elucidate the mechanisms by which bile salts caused improved dissolution of five steroids. They examined wetting, solubilization and diffusivity, separately for each steroid, over a range of bile salt concentrations. The mechanism of dissolution enhancement was

concluded to vary depending on the individual compound, even when the steroids were structurally related. However for the most hydrophobic compound tested, danazol, the main mechanism of dissolution enhancement was concluded by the authors to be solubilization, whereas for the others wetting effects predominated.

The above *in vitro* studies only examined the effect of bile salts on drug solubility and dissolution rate. The influence of lecithin also deserves consideration for a closer representation of *in vivo* conditions. Lysolecithin, the product of phospholipase A activity upon lecithin in the duodenum, enhanced dissolution rates and solubilization of hexestrol, dienestrol and griseofulvin *in vitro*[218]. Although it is a natural progression to simulate *in vivo* conditions by including lecithin with bile salts, several workers have gone a stage further by including products of triglyceride digestion within micellar systems. This may be a better representation of intestinal mixed micelles formed in the lumen of the fed gut.

The formation of bile salt and lecithin mixed micelles in bile has also been studied widely in an attempt to elucidate further the mechanisms by which solubilization may occur. Upon storage in the gallbladder bile becomes concentrated resulting in mixed aggregates in the form of vesicles or micelles. Small[219] originally described the structure of bile salt / lecithin mixed micelles as disc-shaped bimolecular leaflets of lecithin surrounded on their hydrophobic parts by bile salt molecules. The mixed disk model was later proposed by Mazer et al[220] where the disc shaped micelle was described as comprised of a lecithin bilayer interspersed at a constant ratio with hydrogen bonded bile salt dimers. Further bile salt molecules were thought to be arranged as a layer on the outer micelle surface to prevent contact between the lecithin hydrocarbon chains and water. Cholesterol and cholesterol ester was suggested to be incorporated within the lecithin bilayer.

Although this model became widely accepted several studies using different analytical techniques have suggested the mixed micelles to have a flexible, cylindrical structure[221-224] with a radius of approximately 27 Å[221, 224]. A recent theoretical model[225] to describe the solubilization of phospholipid bilayers by micelle forming surfactants also concluded that cylindrical micelles were the most probable structures resulting from aggregation.

The core shell model[224] proposes these cylindrical micelles to have a central core comprised of the lecithin tails arranged radially about the micelle axis, with the cylinder ends capped by bile salts. The micelle perimeter described as the shell includes the lecithin headgroups, bile salt molecules and water of hydration if present. These cylindrical micelles are expected to be formed close to the micellar phase limit in coexistence with premicellar bile salt / lecithin vesicles[226].

Micelles comprised solely of bile salt molecules are associated via hydrophobic interactions between the bile salt hydrophobic faces and by hydrogen bonding of hydroxyl groups. This is possible due to the amphipathic nature of bile salt molecules, which have a side with dominant hydrophobic character and a hydrophilic face formed from hydroxyl groups and a short hydrophilic tail[227]. These bile salt micelles have been reported to have aggregation numbers of approximately 10 and a mean radii of approximately 10 Å[227, 228]. Recent studies confirmed the radius and described a trimer of three bile salt molecules as constituting the building unit of the micellar 7/1 helical structure[229, 230].

The average human bile salt concentration has been reported to range from 10 to 20 mM in the fed and from 4 to 6 mM in the fasted state[231]. Throughout the investigations described in this chapter, (with the exception of section 6.4) a bile salt concentration of 15 mM was selected as an average representation of *in vivo* conditions in the fed state. This concentration is also above the CMC of 2.5 mM quoted for sodium taurodeoxycholate[80], the bile salt used throughout this investigation. Although direct comparison of CMC values is complicated by dependency upon experimental parameters, other workers have reported the CMC of bile salt to decrease upon addition of monoglyceride and / or lecithin[80, 82, 210, 232, 233] to the system. It is therefore a fair assumption that the concentrations of bile salt, lecithin and triglyceride digestion products used throughout this work resulted in micellar solutions.

6.1.1 Outline of this investigation.

The aim of this study was to assess the influence of the major physiological surfactants present in the intestine, namely bile salt and lecithin, upon the solubility of a range of steroids *in vitro*. An enhancement in the solubility of these drugs would indicate a possible increase in their dissolution rate, if administered in the fed state when the level of bile is at its highest.

The solubility values determined were expressed in terms of the extent of solubility enhancement (S/S_o), defined to be the solubility in micellar solution as a fraction of the intrinsic solubility in water. Intrinsic aqueous solubility (S_o) of the steroids was determined in Sörensens phosphate buffer at pH 7 and the results are shown in Table 6.1.

Solubility enhancement was assessed for a range of steroids in relation to:

1. The concentration of bile salt / lecithin (3:1 ratio) mixed micellar solution over a concentration range of 0 to 15 mM bile salt.

2. The hydrophobic nature of the steroid, expressed via an experimentally determined log P_{HPLC} value. A range of ten steroids with varying hydrophobic character were selected for use however log P_{oct} values quoted differed or were not available for all of the steroids chosen. An apparent partition coefficient for each steroid was therefore determined (log P_{HPLC}) using reverse phase high-performance liquid chromatography (RP-HPLC) as described in section 6.10.

3. The ratio of bile salt to lecithin used in the mixed micellar solution.

4. The inclusion of typical products of triglyceride digestion with bile salt to form a mixed lipid / bile salt micellar solution. This approach was taken to increase representation of the physiological fed state.

6.2 Materials

Steroids

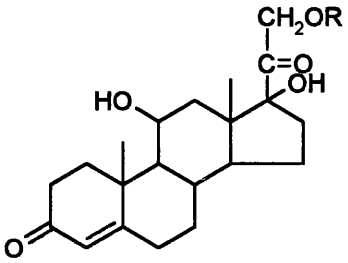
All obtained from Sigma (Poole) and used without further purification

Steroid	Code	Mean $\varepsilon_{\lambda max\ (nm)}$ (litres mole^{-1} cm^{-1})	Standard error as % of mean ε	Intrinsic aqueous solubility (S_o) (mg ml^{-1} / × 10^{-3})
Ethynylestradiol	E-4876	$\varepsilon_{281} = 2175$	8.00	10.96
Hydrocortisone	H-4001	$\varepsilon_{247} = 20050$	0.36	619.88
Hydrocortisone acetate	H-4126	$\varepsilon_{247} = 21767$	3.59	8.36
Hydrocortisone butyrate	H-5270	$\varepsilon_{246} = 20040$	2.46	63.74
Hydrocortisone caprylate	H-6259	$\varepsilon_{246} = 17052$	2.18	1.82
Hydrocortisone valerate	H-5395	$\varepsilon_{246} = 19839$	4.32	17.12
Progesterone	P-0130	$\varepsilon_{244} = 18752$	2.57	0.92
Testosterone	T-1500	$\varepsilon_{247} = 18318$	1.89	12.29
Testosterone acetate	T-1625	$\varepsilon_{242} = 16897$	6.78	0.15
Testosterone enanthate	T-3006	$\varepsilon_{240} = 16988$	2.78	1.28

Table 6.1 Molar absorption coefficient, ε at stated λ_{max} and intrinsic aqueous solubility, S_o of each steroid listed.

Chemical structures of steroids used in this investigation

For R



Hydrocortisone —H

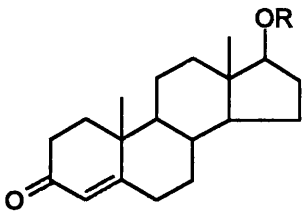
Hydrocortisone acetate —C(=O)CH3

Hydrocortisone butyrate —C(=O)(CH2)2—CH3

Hydrocortisone valerate —C(=O)(CH2)3—CH3

Hydrocortisone caprylate —C(=O)(CH2)6—CH3

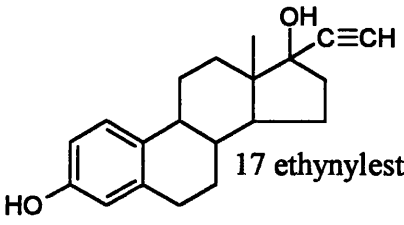
For R



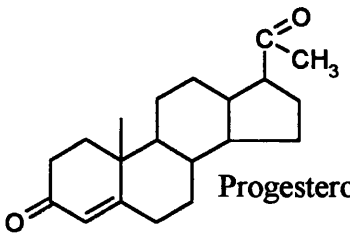
Testosterone —H

Testosterone acetate —C(=O)CH3

Testosterone enanthate —C(=O)(CH2)5—CH3



17 ethynylestradiol



Progesterone

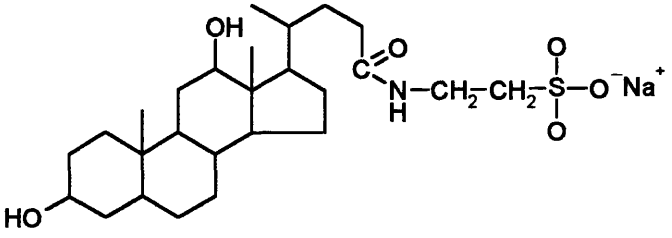
Sörensens phosphate buffer at pH 7

For 1 litre:	Code	Supplier
392 ml Potassium dihydrogen orthophosphate (KH_2PO_4) $^1/_{15}$ M	P-4800	Fisons
608 ml di-Sodium hydrogen orthophosphate dihydrate ($Na_2HPO_4 \cdot H_2O$) $^1/_{15}$ M	S-4450	Fisons
4.411g Sodium chloride (NaCl)	S-9625	Sigma

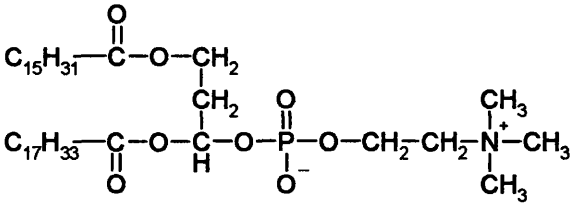
Other materials

		Code	Supplier
Sodium taurodeoxycholate		T-0875	Sigma
L-α-Phosphatidylcholine	(99 % pure from fresh egg yolk)	P-3556	Sigma
Chloroform	(HPLC grade)	C-4966	Fisons
Ethanol absolute	(HPLC grade)	E-0665	Fisons
Methanol	(HPLC grade)	M-4056	Fisons
1-Monoolein		M-7765	Sigma
Oleic acid		O-3879	Sigma

Sodium taurodeoxycholate
referred to in text as "bile salt"



Phosphatidylcholine
referred to in text as "lecithin"



6.3 Method

6.3.1 Preparation of micellar solutions.

A single bile salt (sodium taurodeoxycholate) was selected for use in the micellar solutions to simplify identification of ultraviolet absorbance due to bile salt during analysis of samples. Bile salt micellar solution was prepared by dissolving the appropriate quantity of bile salt in Sörensens phosphate buffer at pH 7. The mixed micellar solution consisted of bile salt (sodium taurodeoxycholate) and lecithin (L-α-phosphatidylcholine) at a ratio of 3:1 in the same buffer. Throughout this work the mixed micellar concentration (expressed in units of mM) refers to the bile salt concentration, with lecithin also present at one third of the bile salt concentration unless otherwise stated.

To prepare the mixed micellar solution appropriate quantities of bile salt and lecithin were dissolved in 20 ml of solvent comprised of methanol and chloroform at a ratio of 2:3. The resultant clear solution was placed in a 500 ml Büchi flask and the solvent drawn off using a rotary evaporator (Büchi Rotavapor R110). The flask was then attached to a vacuum line overnight to ensure removal of any remaining chloroform. The resultant clear film of bile salt and lecithin remaining on the flask surface was subsequently dissolved in an appropriate volume of buffer to give the concentration of bile salt / lecithin mixed micellar solution required.

6.3.2 Determination of steroid solubility in aqueous and micellar systems.

To determine the solubility of the steroids in buffer, bile salt micellar solution and bile salt / lecithin mixed micellar solution, excess steroid was added to approximately 3 ml of potential solvent in a 4 ml capacity clear glass vial with a Teflon lined rubber sealed cap. Each solution was mixed by vortexing (Fisons Whirlimixer) upon addition of steroid to give a saturated suspension, with three replicates prepared for each solution. Vials were placed in a horizontal position to keep drug moving throughout the solution and left for 72 hours in a shaking incubator (New Brunswick Scientific Controlled Environment Incubator Shaker) set at 150 r.p.m., 37°C. After this

equilibration period drug not dissolved was removed from the solution by filtration through a 0.2 μm filter (Acrodisc® non-sterile filters 25 mm, Code no.4496) and the resulting filtrate was retained for assay.

Quantitative determination of the concentration of steroid dissolved in the filtrate was achieved via an ultraviolet spectroscopic method. A double beam instrument (Perkin-Elmer Lambda 3 UV/VIS Spectrophotometer) capable of automatic scanning over a specified wavelength range (200 to 320 nm for this work) was employed.
Absorbance due to bile salt occurred in the same area as the wavelength of maximum absorption (λ_{max}) for the steroids. The spectrophotometer was able to automatically subtract the background absorption due to bile salt from the sample scan up to a bile salt concentration of 6 mM. Dilution of filtrate with buffer was necessary when higher concentrations of bile salt were present. To ensure accurate removal of absorption due to bile salt, the micellar solutions used in the sample and blank needed to be from the same batch and treated in the same manner with regards to dilution. A scan of the blank as a final check ensured complete subtraction of solvent effects from the sample scan throughout the wavelength range of interest.
The absorption reading for each sample was taken at the λ_{max} for the steroid. Dilution of filtrate was performed if steroid in the sample gave an absorbance reading above 0.8, as accuracy for spectrophotometric detection peaks in the range of 0.2 to 0.7[234].
To convert the absorbance reading obtained from each sample into the concentration of steroid dissolved, use was made of the Beer-Lambert Law (Equation 6.1)[234] which describes the relationship between the absorbance of a solution and concentration.

$$\varepsilon = \frac{A}{cl} \qquad \text{Equation 6.1}$$

where ε = The molar absorption coefficient (i.e. absorbance of a molar solution at λ_{max} in a 1 cm path length cell).

A = absorption at λ_{max}

c = molar concentration

l = path length of cell

For each sample the concentration of steroid dissolved was calculated via Equation 6.1 using the absorbance reading, A from the sample and the respective molar absorption coefficient, ε shown in section 6.2. Determination of ε is discussed below.

6.3.3 Determination of the molar absorption coefficient, ε for each steroid.

The following method was used to determine ε for each steroid listed in section 6.2. A standard solution of steroid in ethanol was used to perform a 1:99 dilution into mixed micellar solution (5 mM). The standard solution was prepared so that the dilution produced a micellar solution with an absorption reading of approximately 0.5, the region close to maximum spectrophotometric accuracy[234]. The use of a 5 mM concentration of mixed micellar solution enabled drug to remain dissolved, whilst assay was still possible without prior dilution of the solution.

The absorption reading at the λ_{max} was obtained and used to calculate ε via Equation 6.1. Three replicates were performed for each solution and the average value of ε was determined for each steroid. The resultant values are shown in section 6.2 and were subsequently used in Equation 6.1 to calculate the concentration of steroid dissolved in all assay samples.

ε is known to vary with wavelength however the λ_{max} for each steroid listed in section 6.2 had been demonstrated[235] to remain constant ± 1 nm across a range of mixed micellar solutions with concentrations from 1 to 15 mM. This justified the use of a single ε value (determined in 5 mM mixed micelle solution) for all assay samples with micellar concentrations within this range.

6.4 Influence of the concentration of bile salt / lecithin mixed micellar solution on the extent of solubilization of steroids.

This study was designed to examine if the extent of solubility enhancement shown by the steroids was related to the concentration of bile salt and lecithin mixed micelles in the dissolution media. The solubility of hydrocortisone acetate, hydrocortisone caprylate and progesterone was assessed in buffer and 1, 2, 4, 6, 8, 10, 12, and 15 mM bile salt / lecithin mixed micellar solution as described in section 6.3.2. The

solutions were left for an equilibration period of 24 hours before filtration and subsequent determination of the concentration of steroid dissolved.

6.4.1 Results and discussion.

Figure 6.1 demonstrates the change in solubility enhancement (S/S_o) as a function of bile salt / lecithin mixed micelle concentration expressed in terms of the concentration of the bile salt. Error bars are the standard error of three replicates.
Hydrocortisone acetate and hydrocortisone caprylate (Figure 6.1) show a general trend of increased solubility with an apparent maximum reached at a micellar concentration of 8 mM, although variation across each set of three replicates made this inconclusive. In the case of progesterone a monotonic increase in steroid solubility across the complete range of micellar concentrations was exhibited (Figure 6.1) which probably can be attributed to solubilization of progesterone within the mixed micelles.
Experimental error could possibly account for some variability in the hydrocortisone acetate and hydrocortisone caprylate results. The solubility enhancement ratio determined at the 15 mM bile salt concentration was compared with the S/S_o from an identical experiment where the equilibration time was extended to 72 hours. With an extended period of equilibration an overall increase was seen in the values obtained. The magnitude of the increase (125 % for hydrocortisone acetate, 39 % for hydrocortisone caprylate and a mere 5 % for progesterone) may explain inconsistencies seen for hydrocortisone acetate and hydrocortisone caprylate in Figure 6.1, and as a consequence all future experiments were left to equilibrate for 72 hours.
Several studies have demonstrated a linear increase in steroid solubility with bile salt concentration when the CMC of bile salt was exceeded[217, 236-239]. Although the results obtained here (Figure 6.1) have shown a general trend of increased solubility with concentration of the mixed micelles the relationship was not linear, even above the bile salt CMC of 2.5 mM. This may be due to the experimental procedure used as previously discussed, however another point for consideration is the influence of lecithin upon solubilization of steroids as the studies quoted above involved bile salt micelles.

An investigation[233] examining the solubility of hydrocortisone in the presence of bile salt / lecithin (ratio of 4:1) mixed micellar solution reported a linear increase in solubility for bile salt concentrations in the range of 4 to 15 mM. However an extensive study of the solubility of diazepam[232] in bile salt / lecithin mixed micellar solution over a wide range of ratios and concentration demonstrated that whilst the solubility increase was linear with bile salt alone, the presence of lecithin over the same bile salt concentration range resulted in negative deviation. Results from these two studies suggest the existence of a linear relationship between solubility of drug and the concentration of bile salt and lecithin micelles may be more sensitive to physicochemical properties of the drug than when using bile salt micelles. The presence of lecithin may introduce a different mechanism of solubility enhancement.

6.5 Influence of the hydrophobicity of steroids on micellar enhancement of steroid solubility.

The hydrophobicity of a drug is an important parameter in the relationship between dissolution rate and bioavailability of a drug when administered by the oral route. Thus an increase in the aqueous solubility of a drug via micellar solubilization could provide a means of increasing dissolution rate and eventual bioavailability as already discussed.

A series of steroids were employed to compare the influence of drug hydrophobicity (represented by log P_{HPLC}) upon the extent of steroid solubilization within aqueous micellar solutions. The solubility of each steroid listed in section 6.2 was assessed in buffer, bile salt micellar solution (15 mM) and bile salt / lecithin mixed micellar solution (15 mM) using the method described in section 6.3.2.

6.5.1 Results and discussion.

Figure 6.2 illustrates the mean solubility enhancement (S/S_o) of each steroid as a function of steroid hydrophobicity, represented by the parameter of log P_{HPLC} (determined in section 6.10). The extent of solubility enhancement in both bile salt and mixed micellar solutions (Figure 6.2) increased with steroid hydrophobicity. This

relationship could be predicted as the tendency of a drug to partition within a non-polar phase (the environment of the micelle in this instance) as opposed to remaining in the aqueous phase will increase in line with hydrophobicity of a drug.

The observation of a relationship between the extent of solubility enhancement within surfactant solutions and the hydrophobic nature of the solubilizate has commonly been reported[240]. For example a study by Collett et al[241] examined the partitioning behaviour of a series of substituted benzoic acids into polysorbate 20 micelles as a function of the hydrophobicity of their substituent groups. A linear relationship between the extent of solubilization into micelles and the hydrophobic nature of the substituent groups was taken as an indication that the compounds were solubilized into the lipophilic regions of micelles. In the specific case of steroids Tomida et al[242] established a linear relationship between log P_{oct} and the extent of solubilization within polyoxyethylene lauryl ether micelles for 19 steroids. The correlation was found to improve when the steroids were divided and analysed as separate groups depending upon the presence of a fluorine atom in the molecule.

Indeed the suggested relationship between hydrophobicity of the solubilizate and the extent of solubilization has recently been used by Mithani et al[239] to develop a model for prediction of the extent of solubilization of poorly water-soluble drugs in bile salt micelles. The model uses the drug parameters of log P_{oct} and aqueous solubility. Development of the model involved determination of the solubility of 6 steroids in water and bile salt micellar solution (15mM). This information was used to calculate the solubilization ratio (*SR*) (Equation 6.2), described as the ratio of the solubilization capacity of bile salt for the drug to the solubilization capacity of water for the drug.

$$SR = \frac{SCbs}{SCaq} \qquad \text{Equation 6.2}$$

where

SC_{bs} = solubilization capacity of the bile salt micelles (moles drug / moles bile salt)

SC_{aq} = solubilization capacity of water (moles drug / moles water)

The results[239] showed a linear correlation (Equation 6.3) between log *SR* and log P for the 6 steroids plus a further 5 drugs, despite the lack of similarity between the structure of the drugs, although log P values of the drugs used did not exceed 4.6.

$$\log SR = 2.09 + 0.64 \log P \qquad r^2 = 0.951 \qquad \text{Equation 6.3}^{239}$$

The S/S_o ratio for each steroid in bile salt, displayed in Figure 6.2, was converted into a log *SR* value to enable comparison of results from this investigation with those of Mithani et al[239]. Figure 6.3 illustrates spread of the resultant log *SR* values around the model of Mithani (Equation 6.3). The linear correlation between log *SR* values from Figure 6.2 and log P_{HPLC} is expressed in Equation 6.4 for steroids with log P_{HPLC} values < 5.

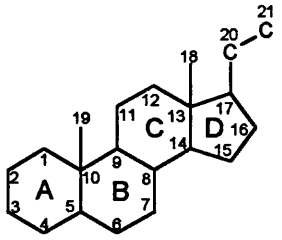
$$\log SR = 2.18 + 0.72 \log P \qquad r^2 = 0.82 \qquad \text{Equation 6.4}$$

Although experimental conditions differed between the two sets of results in that a different bile salt was used, sodium taurocholate for Mithani's investigation and sodium taurodeoxycholate in this work, the data presented here supports the model of Mithani. For drugs with a log P > 5 the linear relationship does not appear to hold although this may be a result of experimental error in determination of the very high log P_{HPLC} values.

The hydrophobic character of a drug appears to be a major influence upon the extent of solubilization although other physicochemical characteristics of a drug may also be involved. Discrepancy between the over prediction and actual solubility of diazepam and phenytoin in bile salt micellar solution[239] using the model of Mithani provides some support to this suggestion.

The molar volume of the solubilizate has been considered in the past to be an influence upon the degree of solubilization on the basis that volume within the micelle for solubilizate is limited. An early study[243] demonstrated inverse proportionality between the volume of hydrocarbons solubilized and molar volume in potassium laurate micelles. However the same relationship does not seem to have been substantiated using bile salt micelles. Miyazaki et al[244] when investigating the

preferential solubilization in bile salt micelles of indomethacin compared with the more lipophilic compound phenylbutazone, concluded molar volume not to be a significant factor. In fact the chemical structure of phenylbutazone was deemed to be of importance with suggested negative repulsive forces between the drug and bile salt. Molecular shape of a drug is another possible influential factor on the extent of solubilization of drug in bile salt micelles. The extent of solubilization of steroidal drugs in various micellar solutions has been discussed by Sjöblom and Ekwall[245] in terms of molecular structure, mainly in relation to the hydrophilicity of the substituent at position 17.



Steroid nucleus to illustrate ring numbering reproduced from reference[245].

Although results indicated higher solubilization to occur with increased substituent hydrophilicity, later work led Sjöblom[246] to state that micellar solubility is determined by the structure of the steroid molecule as a whole, based on the following criteria:

1. Hydrophilic substituents do not unconditionally increase micellar solubility, as orientation of the steroid in the micelle may depend on balance of hydrophilicity between rings AB and CD (see structure above).
2. Position of the hydrophilic substituent is important.
3. Suitable hydrocarbon substituents increase solubility in the micelle.

The dominant substituent on the steroid frame in terms of solubilization enhancement may thus have influence with regards to orientation of the steroid molecule in the micelle. Micellar solubilization has been proposed to occur by three mechanisms[247], incorporation of solubilizate into the hydrocarbon core of the micelle, orientation of the solubilizate into the palisade layer of the micelle in the same manner as the

surfactant, or by solubilizate adsorption onto the micelle surface. Early studies involving space filling models[236] demonstrated the possibility of the steroid, 19-nortestosterone being solubilized within the micelle interior. Slightly later work involving NMR[237] suggested the solubilization mechanism of testosterone involved complex association of bile salt and steroid molecules to form mixed micelles.
The most likely mechanism by which steroids are solubilized in bile salt micellar solution appears to be via orientation into the palisade layer of the micelle, with polar functional groups on the steroid frame maintaining some contact with the aqueous surroundings. Cai et al[248] determined an activity coefficient value to quantify intermolecular interactions of steroid with solvent (water, bile salt and mixed bile salt / lecithin micellar solution). When using water as the solvent the activity coefficient was noted to differ between the steroids investigated. The activity coefficient decreased with increasing numbers of hydroxyl groups on the steroids and reflected their respective positions and hence ability to hydrogen bond with solvent. The same pattern was seen for the micellar solutions, albeit with reduced activity coefficient values due to the more favourable hydrophobic environment of the micellar solution. Demonstration of the same pattern suggests polar interactions between steroid and solvent still occur to a certain degree in micellar solution and hence total incorporation of the steroid molecule within the micelle interior is questionable.
In the case of the steroids examined in this investigation the position or nature of functional groups on the steroid frame appears to have altered the solubilization enhancement of steroids with similar hydrophobicity. The hydrocortisone esters, despite a general trend of increasing solubilization with lipophilic character, did not become solubilized to the same degree as other steroids tested with similar log P_{HPLC} values (Figure 6.4). From examination of steroid structures illustrated in section 6.2 the hydrocortisone esters differ mainly in the possession of a hydroxyl group at position 11, and also at position 17 together with a carbonyl group. The hydroxyl groups may result in orientation of the hydrocortisone steroid frame near the periphery of the micelle palisade layer due to preferential hydrogen bonding. Polar and hydroxyl groups on the bile salt and steroid may form H-bonds with the solvent and dominate over van der Waal forces between the steroid frame and hydrophobic areas

of the bile salt and lecithin molecules. This may result in a less efficient arrangement of the steroid frame within the micelle structure and hence decreased solubilization. For the testosterone series when the length of the hydrocarbon side chain was increased from 2 carbons on testosterone acetate (log P_{HPLC} 4.71) to 7 on testosterone enanthate (log P_{HPLC} 9.05) the extent of solubilization increased in bile salt / lecithin micelles but fell slightly in bile salt micelles (Figure 6.2). The decreased solubilization in the bile salt micellar solution did not reflect the increase in hydrophobicity of the drug and may perhaps be explained by spatial restriction within the micelle area. The smaller bile salt micelles may allow solubilization of a finite number of steroid nuclei, with the bulky hydrophobic side chain of testosterone enanthate restricting further solubilization. The longer side chain could be folding into the hydrophobic region of the micelle leaving less space for incorporation of further testosterone nuclei which the larger bile salt / lecithin micelle is able to accommodate.

6.6 Influence of lecithin on the solubilization of steroids by bile salt micelles.

The correlation between steroid hydrophobicity and extent of solubilization appears to be mainly unaffected by the presence of lecithin, as bile salt micellar solution and bile salt / lecithin mixed micellar solution had similar solubilizing effects for the steroids tested (Figure 6.2); marginal differences were apparent between results from the two solutions but no obvious trend was evident to explain these.

The correlation (Figure 6.2) had been demonstrated when using a bile salt to lecithin ratio of 3:1, selected to represent intermediate conditions found in the small intestine. The ratio in healthy human subjects has been reported to range from 2.5:1 up to 5:1[249]. To further examine the influence of lecithin upon solubilization enhancement of steroids, a study was performed using a wider range of possible physiological bile salt to lecithin ratios.

Solutions were prepared as per section 6.3.1 to produce bile salt / lecithin mixed micellar solutions with the following ratios: 10:1, 5:1, 3:1 and 2:1, with the bile salt concentration fixed at 15 mM in each case. The solubility of progesterone and

testosterone acetate was determined in each of the above solutions using the method described in section 6.3.2.

6.6.1 Results and discussion.

Figures 6.5 & 6.6 illustrate the dependency of mean solubility enhancement (S/S_o) on the bile salt to lecithin ratio of mixed micellar solution for progesterone and testosterone acetate respectively. For both drugs solubility enhancement increased as the proportion of lecithin to bile salt in the mixed micellar solution was raised. Bile salt micelles were equal to or more effective in terms of solubility enhancement than bile salt / lecithin mixed micelles up to a ratio of 3:1.

Rosoff et al[232] carried out a similar investigation of the solubilization of diazepam (log $P \approx 3$[250]) in bile salt / lecithin mixed micelles with identical ratios (10:1, 5:1, 3:1 and 2:1) to those tested above. These workers also reported the solubilization capacity of a mixed micellar solution for diazepam to increase with the proportion of lecithin present. The results were proposed to be a consequence of increased micellar volume, based on a study by Shankland[251] which demonstrated (via molecular weight determination) the size of a mixed micelle to increase with the lecithin to bile salt ratio. Micelles with a low ratio of lecithin to bile salt would therefore have a smaller volume and reduced capacity to solubilize diazepam than larger micelles with a higher proportion of lecithin. Sufficient lecithin is necessary to produce a bile salt / lecithin mixed micellar system with no pure bile salt micelles remaining[228]. However an increase in lecithin above this concentration should not increase the micelle radius assuming the core shell model is correct, as the cylinder radius is determined largely by the length of the lecithin tail[221].

Rosoff et al[232] also stated that within the physiological concentration range of bile (< 40 mM), mixed micelle formation with any ratio of lecithin always resulted in an increase in diazepam solubility above the concentration in bile salt alone. This conflicts with results from these investigations possibly reflecting the dependence of solubilization upon physicochemical characteristics of individual drugs.

A later study[233] examining the ability of lecithin, in a 1:4 ratio with bile salt, to modify the mechanism of dissolution for hydrocortisone demonstrated a marginal increase in

solubility in the mixed micellar system compared to bile salt alone. The initial dissolution rates indicated inclusion of lecithin strongly suppressed the ability of bile salt to wet the hydrocortisone powder, with increased dissolution mediated through solubilization effects from lecithin. Two other steroids, betamethasone and danazol were also studied[82] to examine the effect of bile salt and lecithin (4:1 ratio) on dissolution rate. For both drugs inclusion of lecithin increased solubility compared to the value in bile salt alone (15 mM), with the highly lipophilic danazol (log P 4.53) showing a solubility value at least 5 times higher. The large increase in the solubility of danazol was suggested to reflect the ability of the danazol molecule to enlarge the diameter of the bile salt / lecithin micelles and promote swelling resulting in higher aggregation numbers than usual.

Unlike danazol[82], testosterone acetate in this study did not exhibit a dramatic increase in solubility when lecithin was included with bile salt (Figure 6.6). This was despite the use of the same concentration of bile salt in both studies and the similar log P values of the drugs (4.85 cf. 4.53). The extended ring structure of danazol[82], closer in resemblance to taurocholate than testosterone acetate, may aid efficient integration with bile salt in the perimeter of the bile salt / lecithin micelle structure. Consequently higher solubilization may be possible than with a conventional steroid frame, regardless of lipophilic character of the solubilizate.

6.7 Influence of triglyceride digestion products upon enhancement of the solubility of steroids by bile salt micelles.

To improve representation of micelles likely to be formed in the physiological fed state, typical products of triglyceride digestion, namely fatty acids and monoglyceride, were added to the bile salt and bile salt / lecithin micellar solutions. The resultant bile salt / mixed lipid micelles would thus provide a closer indication of the solubility enhancement of steroids likely to be achieved *in vivo* within intestinal mixed micelles. The molarity of the lipolytic products included in the micellar solution was based on complete digestion of a minimal quantity of triglyceride (0.5 % $^{w}/_{v}$). Oleic acid and 2-monoolein (ratio 2:1) were selected as representative of typical lipolytic products

formed by *in vivo* digestion of a common dietary triglyceride, glycerol trioleate. For economic reasons 2-monoolein was substituted in this investigation by 1-monoolein. The bile salt / mixed lipid micellar solution was prepared in the same manner as described for the bile salt / lecithin mixed micellar solution (section 6.3.1) with 0.283 mM of 1-monoolein and 0.565 mM of oleic acid added to the chloroform / methanol solvent at the same time as the bile salt. Upon addition of buffer to the lipolytic products and bile salt (15 mM) a clear micellar solution was produced. However the same components with inclusion of lecithin (5 mM or 1 mM) produced turbid solutions which were discarded from the study as unsuitable for assay using an ultraviolet technique.

The solubility of hydrocortisone acetate, hydrocortisone valerate, progesterone and testosterone acetate was determined in the 15 mM bile salt / mixed lipid (monoolein & oleic acid) micellar solution using the method described in section 6.3.2.

6.7.1 Results and discussion.

Figure 6.7 illustrates the mean solubility enhancement in bile salt / mixed lipid micellar solution, bile salt / lecithin mixed micellar solution and bile salt micellar solution for the steroids indicated. The mixed lipid / bile salt micelles significantly increased solubility enhancement for three out of the four steroids examined. Lipid inclusion did not increase the solubility enhancement of hydrocortisone acetate above that determined in the presence of lecithin.

The extent to which mixed lipid micellar solution enhanced solubility (relative to the other micellar solutions tested) appeared to increase with the log P of the drug, although the range of steroids tested was not wide enough to confirm this. The mixed lipid micelles could be assumed to be more lipophilic than the bile salt / lecithin micelles, with the bile salt micelles the least lipophilic. The enhancement of solubility may therefore be explained by increased partitioning of the steroids with higher hydrophobicity into the more lipophilic micelles.

An investigation into the effect of bile salt / fatty acid micelles on the extent of solubilization of the lipophilic drugs, griseofulvin, dantrolene and ketoconazole revealed solubilization to increase in line with drug hydrophobicity[79]. As with this

work when the results (of dantrolene) were compared with the extent of solubilization in bile salt / lecithin mixed micelles, the bile salt / fatty acid micelles had the greater effect. For clofazimine, described as a highly lipophilic drug, mixed bile salt / fatty acid micelles increased drug solubility compared to solubility achieved in bile salt micelles[252]. To some extent these results are conflicting with those from Luner et al[253] who stated that solubilization of gemfibrozil within bile salt / lecithin micelles was additionally enhanced by the presence of monoglyceride and unaffected by fatty acid salts.

The mechanism by which addition of lipolytic products to bile salt micelles enhances drug solubility may involve the larger size of the bile salt / mixed lipid micelle allowing incorporation of an increased number of drug molecules. Alternatively a different mechanism of solubilization may be occurring whereby, due to the more hydrophobic nature of the micelle, drug molecules are also able to partition into the hydrophobic interior rather than undergoing specific orientation in the palisade layer.

Interestingly for the least hydrophobic drug tested in this investigation, hydrocortisone acetate, solubility enhancement was reduced slightly compared to results using the bile salt / lecithin micellar solution (Figure 6.7). When an early study[210] noted solubility of the poorly water-soluble drug, hexestrol to decrease in intestinal bile salt mixture upon incorporation of lipid additives, the workers proposed competition between solubilizate and lipid additives for space in the micelle hydrocarbon interior to be responsible. Alternately the increased hydrophobic character of the micelle due to additional lipids may not encourage partitioning of drugs unless they display a certain degree of hydrophobicity.

6.8 Physiological relevance of this investigation.

These *in vitro* investigations have demonstrated the ability of the major physiological surfactants found in the intestine, bile salts and lecithin, to increase the solubility of each steroid tested above their apparent aqueous solubility values. Upon arrival in the intestine therefore these hydrophobic drugs should theoretically dissolve to a greater extent in the presence of bile released from the gallbladder.

In vitro the concentration of bile salt / lecithin micelles appears to have a direct influence upon the degree of solubility enhancement of a drug, as demonstrated in this work for progesterone and reported in the literature by other workers[232, 233]. *In vivo* bile salt concentration is low (3-5 mM) in the fasted state and the solubility enhancement of drugs can be assumed to be minimal, although the CMC of bile salt / lecithin micelles is normally exceeded. Stimulation of bile release in the fed state serves to increase micelle numbers, rather than promote micelle formation[4], thereby increasing the solubilizing capacity of the GI tract. As food is the stimuli for release of bile from the gallbladder it follows that a high level of solubility enhancement is more likely upon dosage in the fed state. In the case of other lipids present in the fed state such as fatty acids and monoglycerides, when incorporated with bile salt the consequential larger volume of the intestinal mixed micelle may further enhance solubilization of drug. Once drug molecules are solubilized within a bile salt / lecithin / mixed lipid micelle they should remain in a soluble state ready for absorption across the intestinal membrane, although it should be remembered that micelles are dynamic structures.

At the stage of transfer of drug from the micelle across the UWL for absorption into enterocytes experimental work has demonstrated a potential disadvantage of drug solubilization within micelles. Intestinal absorption of the poorly water-soluble drug, albendazole, was noted in animal studies to be decreased in the presence of bile salt / fatty acid micelles when compared to absorption when using bile salt alone in the same concentration[83]. The inclusion of fatty acid was proposed to have increased micellar solubilization of drug and reduced the rate of micellar dissociation compared to that exhibited by bile salt micelles, with a resultant decrease in absorption.

In vitro studies[254] demonstrated that despite an increase in griseofulvin solubility in bile salt and lecithin mixed micelles compared to bile salt micelles the dissolution rate did not increase in line with solubility. As dissolution rate depends on solubility and the diffusion coefficient, a decreased diffusivity rate of the micelle due to an increase in the micellar size caused by lecithin was suggested to be responsible. The diffusivity of danazol[82] was dramatically decreased when solubilized within bile salt / lecithin mixed micelles however dissolution rate was still enhanced. A possible explanation is the higher amount of drug per micelle may offset the decrease in diffusivity rate compared to that of free drug.

During studies of cholesterol dissolution in bile salt / lecithin micellar solution a negatively charged interfacial barrier was suggested to exist between the dissolution fluid and the surface of cholesterol[255]. Despite an increase in solubility the resulting electrostatic repulsive forces acted to decrease dissolution, suggesting that for extremely lipophilic drugs surface chemical effects could reduce the dissolution rate of drug in micellar solutions[4].

In general micellar solubilization of drug appears to be advantageous in terms of increasing the dissolution rate of a hydrophobic drug, which is usually the rate limiting step to bioavailability. However from the above examples it should be remembered that the overall result of solubilization and the effect upon dissolution rate requires consideration on an individual basis for each drug.

6.9 Conclusions

This series of investigations demonstrated the ability of bile salt / lecithin / mixed lipid micellar solutions to increase the apparent solubility of the steroids tested compared to their aqueous solubility. The degree of solubility enhancement was dependent on micellar constituents and concentration and also increased in extent with increasing steroid hydrophobicity. Other factors are also involved as the relationship between hydrophobicity of the steroid and degree of solubilization is not linear. These could include the ease with which a particular drug structure can undergo specific spatial orientation into the palisade layer of the micelle.

Variation in the bile salt to lecithin ratio in mixed micelles has demonstrated that lecithin can have an inhibitory effect on solubility enhancement when included in low proportions compared to bile salt alone, but is able to increase steroid solubility when present in a sufficient high concentration, presumably by swelling the micellar volume.

Inclusion of mixed lipids in bile salt micelles also showed an increase in steroid solubility, however these results would have been a more useful representation of *in vivo* conditions if the micelles had also contained lecithin.

The loss of clarity upon inclusion of lecithin in the mixed lipid / bile salt system was probably a result of an increase in the mixed lipid to bile salt ratio. Visual turbidity has been quoted as corresponding to a particle diameter of > 1000 Å[256] which

encompasses the size range of unilamellar vesicles. This suggests these may have been formed in the turbid solution along with mixed lipid / bile salt micelles. Together these structures represent the main phases of lipolytic product dispersal formed *in vivo*, and the turbid medium could thus be considered to simulate the fed state for further drug solubility studies. With development of a suitable method to enable analysis of the turbid solution such as HPLC, the influence of steroid log P upon solubility could be further assessed in a physiological representative media.

Choice of bile salt is another consideration regarding physiological representative media as several studies [232, 236, 244] have demonstrated the degree of drug solubilization to differ slightly with bile salt. The bile salt used throughout this investigation was a conjugated form of deoxycholic acid which constitutes only 15 % of the bile acids present in the human[257]. However the relevance of bile salt used remains questionable as Bates et al[210] concluded results from solubilization of drug in a simulated intestinal bile salt mixture were comparable with those from individual bile salts.

The potential of bile salt / lecithin micelles, released during the digestion of lipid, to improve the bioavailability of drugs is based on the assumption that an increase in the solubility of a drug will increase dissolution rate via the Noyes-Whitney equation (Equation 7.1). The influence of bile salt and lecithin upon dissolution rate has also been discussed in terms of increased effective surface area via wetting[240]. An overview of the literature cited in this chapter suggests the dominant mechanism by which surfactants may enhance or even decrease the dissolution rate of a drug is dependant on physicochemical properties of the drug in question.

6.10 Experimental determination of log P_{HPLC} values for steroids used in this investigation.

6.10.1 Introduction

A quantitative measure of hydrophobicity was required to enable examination of the solubility enhancement of each steroid in aqueous micellar solution in terms of steroid hydrophobicity. The most commonly used parameter of drug hydrophobicity is the log of the partition coefficient (log P_{oct}),[258] defined as the ratio of a neutral unionised substance present in octanol to that in water; a system thought to reflect partitioning of drug between lipid membranes and extracellular fluids[259]. Log P_{oct} values from the literature were only available for five of the ten steroids selected for the solubilization study and those for which figures were quoted often had several different values depending on the source of reference. An alternative method was thus required to rank the steroids in order of hydrophobicity.

Traditionally log P_{oct} has been measured using octanol and water phases via the shaking flask method. Difficulties such as prolonged equilibration times and low aqueous solubility in the aqueous phase are encountered with this method if the log P_{oct} is greater than four[260]. There is also the requirement for a relatively large amount of pure, stable compound[261]. An alternative approach to direct measurement of partition coefficients involves systematic calculation to estimate hydrophobicity. The Hansch and Leo's hydrophobic substituent constants[262],π, and Rekker's hydrophobic fragmental constants[250],f, are methods based on findings of Fujita et al[258] that the log P_{oct} value of a solute is composed of the sum of individual contributions of functional groups which make up the solute. However this is not always the case if a substituent group causes steric or electronic effects within the parent molecule which can lead to inaccuracies in a calculated log P_{oct} value[258] when compared to a value determined experimentally.

The use of RP-HPLC for the determination of partition coefficients was suggested in 1974 by Haggerty and Murrill[263] on the basis that chromatography involves a partitioning process. A direct relationship can therefore be expected between the retention time of a compound within the chromatographic system and partitioning

behaviour described by log P_{oct}. The general procedure to determine log P_{oct} values via RP-HPLC (reviewed by Terada[264] and Lambert[265]) involves experimental assessment of the retention time of reference compounds, with known log P_{oct} values, in a chromatographic system. The retention time is then used to calculate the capacity factor, k' (defined by Equation 6.5) which describes affinity of the compound for the stationary phase.

$$k' = \frac{(t_R - t_o)}{t_o} \qquad \text{Equation 6.5}$$

where t_R = retention time of sample compound.

t_o = retention time of an unretained reference compound.

A regression line between k' and the known log P_{oct} values according to Equation 6.6 allows construction of a calibration curve.

$$\log k' = a + b \log P_{oct} \qquad \text{Equation 6.6}$$

where a and b = constants characteristic of a particular chromatographic system.

The log k' of the test compound can then be measured under the same experimental conditions, with the corresponding partition coefficient value being read from the calibration curve. As there is not a theoretical relationship between log k' and log P_{oct} the partition coefficient determined is an apparent parameter related to it and is generally referred to as log P_{HPLC}.

For log P_{HPLC} to reflect partitioning behaviour seen with the shake flask octanol / water method as closely as possible it is important to select a stationary phase which mimics the behaviour of octanol. Silica gel has been successfully coated with n-octanol to give a stable chromatographic system[266] with the resulting linear

relationship between log P_{oct} and log k' possessing a gradient very close to unity, indicating a direct relationship between the two terms. However the mobile phase used to prevent solubilization of n-octanol from the stationary phase is n-octanol saturated water, which does not allow elution of hydrophobic compounds with a log $P_{oct} > 3$ within reasonable retention times[267]. Glyceryl coated controlled pore-glass beads is another stationary phase which enables accurate determination of log P_{oct} values up to 6[268]. This system has the added advantage that the linear relationship between log k' and log P_{oct} has a slope of 2.26, which reduces retention time in relation to log P_{oct}.

The most commonly used hydrocarbon stationary phase in RP-HPLC is octadecyl silica (ODS) which is composed of octadecyl groups covalently bonded to the silanol groups of silica gel. This type of column can be used to determine log P_{oct} values of up to at least 6[269] with good correlation between log k' and log P_{oct}, especially for a homologous series of compounds[264]. An organic modifier such as methanol is added to the mobile phase to aid elution of the compound from the column and thus reduce retention times, as an increase in organic solvent concentration will decrease solute affinity for the stationary phase.

A linear relationship, expressed in Equation 6.7, has been demonstrated between log k' and methanol concentration in the mobile phase, over a limited range of methanol concentrations[270].

$$\log k' = \log k'_o + mC \qquad \text{Equation 6.7}$$

where k'_o = the capacity factor in absence of methanol from the mobile phase

C = concentration of methanol (% $^{v}/_{v}$)

The k' values for a series of compounds can be correlated with log P_{oct} if the same organic solvent concentration is used for each determination via Equation 6.6. Alternatively the linear relationship expressed in Equation 6.7 can be used to obtain

the log k'_o value by extrapolation to zero percent organic solvent in the mobile phase[264].

For this study RP-HPLC using an ODS stationary phase and methanol / water for the mobile phase was the method felt best suited for determination of the log P_{HPLC} values of the ten steroids, due to their suspected wide range of hydrophobicity. Log k' values were determined for each steroid using a range of methanol / water concentrations in the mobile phase. Log k'_o values were then calculated for each steroid by extrapolation of the relationship between log k' and methanol concentration in the mobile phase according to Equation 6.7. Log k'_o values determined showed a significant correlation with literature log P_{oct} values available, thus allowing estimation of the log P_{HPLC} values of steroids for which log P_{oct} values from the literature were not found.

6.10.2 Experimental

Equipment

HPLC system

Pump	LDC-Milton Roy constaMetric® 3000 solvent delivery system
u.v.detector	Milton Roy spectroMonitor® 3100 ultraviolet detector
Stationary phase	Microsorb Short One C_{18} Code 80-205-C3
Mobile Phase	Methanol HPLC grade (Fisons Code no.M/4056)
	Ortho Phosphoric Acid (Fisons Code no.O/0450)
	(minimum 85 % pure H_3PO_4)
Retention times	Spectra-Physics SP4100 computing integrator
HPLC conditions	
	flow rate 1.5 ml / minute
	u.v.detection wavelength 240 nm
	performed at room temperature
	injection loop volume 20 $\mu\ell$

Method

Steroids (listed in section 6.2) were dissolved in methanol at an approximate concentration of 0.2 mg / ml and injected onto the column. The mobile phase consisted of a mixture of distilled water and methanol with methanol concentration expressed in terms of % $^{v}/_{v}$. The pH of the mobile phase was adjusted to 2.0 with phosphoric acid to prevent acid dissociation of the steroids. Retention times were assessed for each steroid using a minimum of 5 different methanol concentrations in the mobile phase. The methanol concentration in the mobile phase varied from 40 to 90 % in 5 % steps, the range for each steroid being chosen so that the compound exhibited a retention time of 30 minutes or less.

6.10.3 Results

Retention times (t_R) recorded for each steroid were the mean of at least three RP-HPLC measurements performed using the same concentration of methanol in the mobile phase. Injection of pure methanol onto the column showed an identical retention time with all mobile phase concentrations and was used to represent retention time of an unretained reference compound, (t_o) for the system. The resulting data (t_R and t_o) enabled calculation of the capacity factor (log k') values via Equation 6.5 for each steroid at each respective concentration of methanol used. Figures 6.8a-b illustrate the dependence of log k' on the concentration of methanol in the mobile phase. For each steroid log k' decreased linearly with increase in methanol concentration in the mobile phase, indicating organic modifier had lessened affinity of the solute for the stationary phase and hence decreased retention time. Demonstration of a linear relationship between log k' and the concentration of organic modifier enabled the use of Equation 6.7 to obtain the capacity factor in the absence of methanol from the mobile phase, $\log k'_o$. The log k'_o value was calculated for each steroid by extrapolation of the respective plot to zero percent methanol via linear regression analysis and the results are shown in Table 6.2. The correlation coefficients were all higher than 0.996.

To construct a calibration curve as described by Equation 6.6 between log P_{oct} and experimental $\log k'_o$ values, known log P_{oct} values were required. Literature

sources[242, 259, 262, 271.] were found to quote up to seven different log P_{oct} values for some steroids (listed in Table 6.2). This was probably due to the use of various methods and the inherent difficulties associated with log P_{oct} determination as already discussed. To overcome this linear regression analysis was carried out on the log P_{oct} values from each reference source individually and when combined and the results are shown in Table 6.3. Combined log P_{oct} values were selected for construction of the calibration curve as higher statistical confidence was possible in the resultant regression line due to the greater statistical population (assuming random error). The intercept and gradient values from the combined literature log P_{oct} regression line (Table 6.3) were substituted into Equation 6.6 resulting in Equation 6.8.

$$\log k'_o = \underset{(0.1774)}{2.3199} + \underset{(0.0599)}{0.5031}\ \log P_{oct} \qquad \text{Equation 6.8}$$

Substitution of the experimentally determined $\log k'_o$ value for each steroid into Equation 6.8 thus enabled calculation of log P_{HPLC} for each steroid and the resultant values are shown in Table 6.2. Figure 6.9 displays the calibration curve constructed from the combined literature log P_{oct} values, together with the determined log P_{HPLC} value for each steroid (all listed in Table 6.2).

6.10.4 Discussion

For this work the $\log k'_o$ value has been used as a measure of chromatographic retention. This parameter is independent of organic modifier effects, represents well shake flask partitioning and has dependence upon solute structure and function[272]. As methanol is required in the mobile phase to aid solute elution, $\log k'_o$ has to be obtained by extrapolation of the observed linear relationship between log k' and methanol concentration. Whereas this relationship remains linear between 30 and 70 % methanol[273] outside of this range curvature has been observed which is more accurately described by the quadratic expression[274] shown in Equation 6.9.

$$\log k' = AC^2 + BC + \log k'_o \qquad \text{Equation 6.9}$$

The use of methanol as organic solvent has been shown to give less curvature than other organic modifiers[275] and will thus minimise error in linear extrapolation to $\log k'_o$, although even slight curvature can result in deviation from true $\log k'_o$ values. An alternative method for determination of log P_{oct} is the use of a log k' value at a fixed concentration of methanol via Equation 6.6. This approach, in addition to convenience has been found to give good correlation[264, 276], which was improved further upon the use of lower concentrations of organic solvent in the mobile phase (< 30 %)[270].

In this study the broad range of lipophilicity exhibited by the steroids meant that a log k' value for all the compounds when using the same concentration of methanol in the mobile phase was not available. In view of this fact, in combination with the reduced curvature reported for methanol, linear extrapolation to $\log k'_o$ was used. It could be argued that accuracy of the $\log k'_o$ values may be improved by quadratic extrapolation to zero per cent methanol. A study[269] comparing linear and quadratic extrapolation to $\log k'_o$ upon the same experimental data set found close correlation between the two values for polar compounds, however for less polar compounds the quadratic values were higher. This was proposed to be a result of the extensive extrapolation required due to the higher methanol concentrations required for elution of hydrophobic compounds. As the steroids used in this study were hydrophobic in nature and the data obtained appeared linear this gave further support to the use of linear extrapolation to determine $\log k'_o$.

A further influence which may affect the linear relationship expressed in Equation 6.6 when using ODS as the stationary phase appears to be the ability of test compounds to form hydrogen bonds with either the stationary or mobile phase. Studies by Miyake et al[270, 277] examined the relationship between log k' and log P_{oct} using the same chromatographic system with compounds differentiated according to hydrogen bonding ability. Good correlation was observed within the same hydrogen bonding class which was reduced when all of the compounds were examined together. Addition of hydrogen bonding terms to Equation 6.6 gave improved correlation for a wide range of compounds[277] however their effect reduced with the concentration of methanol used to determine log k. The workers concluded that hydrogen bonding

terms may not be required for correlation with $\log k'_o$ as in the absence of methanol hydrogen bonding was small or negligible.

In view of the use of $\log k'_o$ for correlation with log P_{oct} for the steroids investigated in this study and the similar functional groups and hydrogen bonding capabilities of the steroids it was not felt necessary to involve hydrogen bonding terms when evaluating these results.

In further support of the log P_{HPLC} values determined for steroids in this study the parameters of the chromatographic system used were examined. According to Terada[264] the gradient of the regression line between log k' and log P_{oct} for Equation 6.6 will improve accuracy of log P_{oct} if the value is approximately 0.5. For the system in this work the gradient had an excellent value of 0.503. In addition the range of log k' values which can be determined accurately by HPLC were stated to fall within -0.3 to 2.5[264]. For this study the experimental values of log k' also complied to this requirement, ranging between -0.155 and 1.93.

6.10.5 Conclusions

Upon comparison of the experimental log P_{HPLC} values determined here with the quoted literature log P_{oct} values all were found to be within reasonable range of the literature values (Table 6.2). For hydrocortisone caprylate and testosterone enanthate, which are clearly very hydrophobic compounds from their retention behaviour, the log P_{HPLC} values determined were extremely high. It is difficult to have confidence in these values due to the large degree of extrapolation involved in their determination however the linearity of the relationship between log k' and methanol concentration provides some support to the results.

The determination of log P_{HPLC} has provided, by means of chromatographic retention behaviour, a parameter for ranking the series of steroids in order of hydrophobicity. Log P_{HPLC} values enabled correlation between steroid solubility enhancement (via micellar solubilization which is an interfacial partitioning process) and steroid hydrophobicity (section 6.5). In some ways the direct use of log k'_o as a hydrophobicity parameter may be a closer representation of steroid partitioning into micelles whereas a true log P_{oct} value reflects partitioning between bulk phases.

Steroid	Log k'_o	Literature log P_{oct}	Range of literature log P_{oct} values	Experimental Log P_{HPLC}
Hydrocortisone	3.0947 (0.113)	1.53[a] 1.61[a] 1.68[a] 1.78[b] 1.55[c] 1.53[d] 1.93[d]	1.53-1.93	1.54
Hydrocortisone acetate	3.6995 (0.068)	2.67[b] 2.19[c] 2.37[d] 2.70[d]	2.19-2.70	2.74
Testosterone	3.9246 (0.256)	3.31[a] 3.31[a] 3.29[c] 3.31[d] 3.32[d]	3.29-3.32	3.19
Ethynylestradiol	3.9802 (0.232)			3.30
Hydrocortisone butyrate	4.1229 (0.181)	3.63[d]	3.63	3.58
Progesterone	4.3807 (0.230)	3.70[a] 4.60[b] 3.87[c] 3.87[d] 4.01[d]	3.70-4.60	4.10
Hydrocortisone valerate	4.4366 (0.175)			4.21
Testosterone acetate	4.6873 (0.161)			4.71
Hydrocortisone caprylate	6.1274 (0.298)			7.57
Testosterone enanthate	6.8726 (0.305)			9.05

Table 6.2 For the steroids listed:

Experimental log k'_o values from linear extrapolation of capacity factors via Equation 6.7. (Values in parentheses = 95 % confidence limits).

Literature log P_{oct} values (a[262], b[271], c[242] and d[259]).

Predicted log P_{HPLC} values via Equation 6.8.

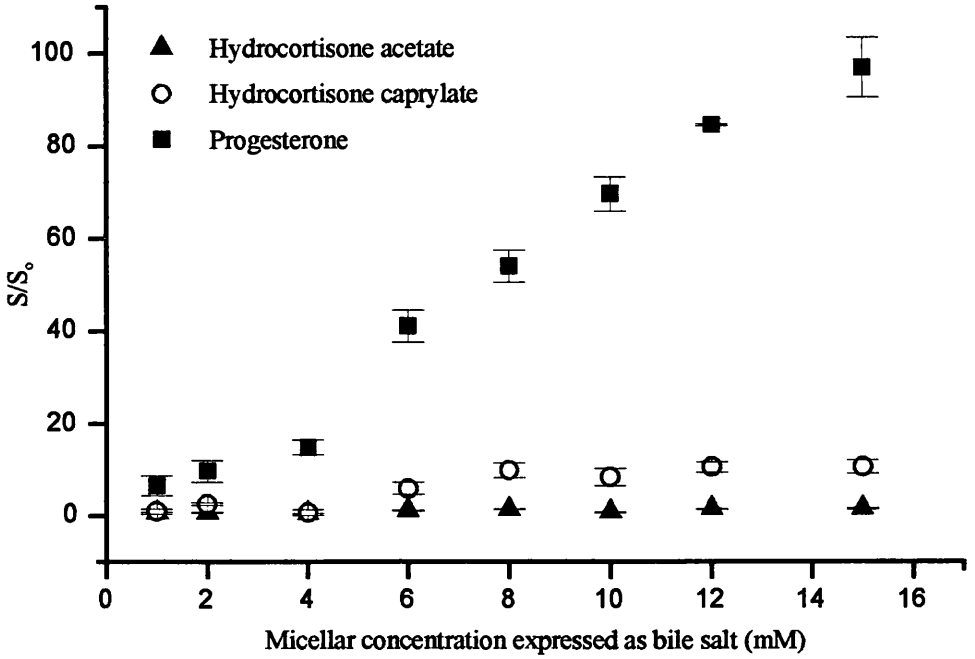
Literature Log P_{oct} values	Gradient	Intercept	r^2	n
a[262]	0.5469	2.2056	0.9727	6
	(0.1273)	(0.3421)		
b[271]	0.4394	2.3993	0.9695	3
	(0.9902)	(3.2065)		
c[242]	0.4907	2.4378	0.9254	4
	(0.4237)	(1.2171)		
d[259]	0.5303	2.242	0.9491	9
	(0.1097)	(0.3376)		
All values combined	0.5031	2.3199	0.9689	22
	(0.0599)	(0.1774)		

Table 6.3 Correlation statistics for literature values of log P_{oct}.

r^2 = correlation coefficent squared.

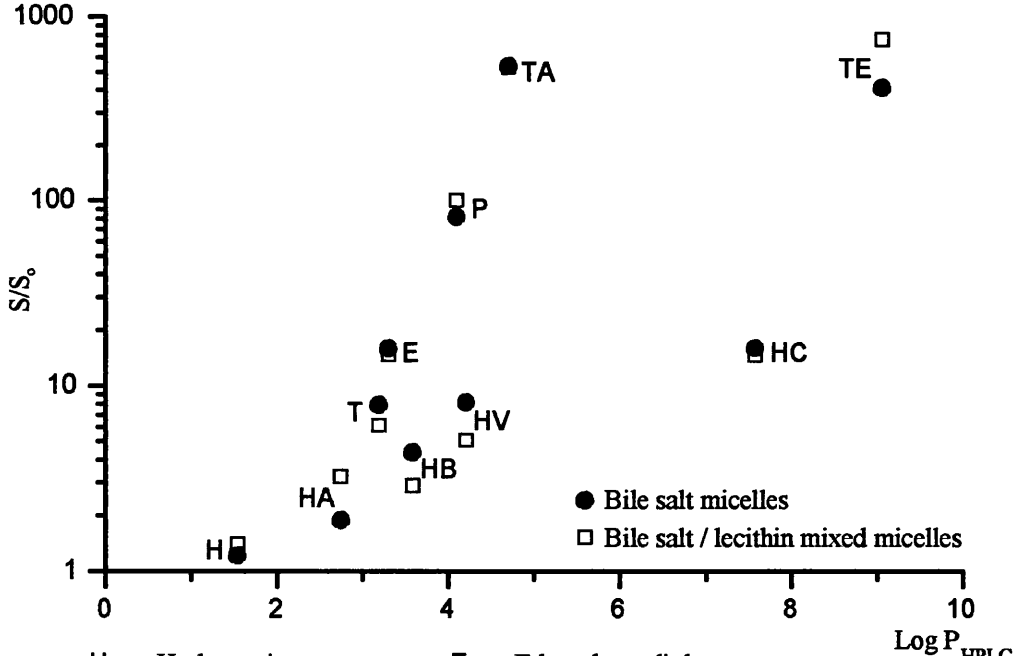
n = no. of observations.

Figure 6.1 Solubility enhancement (S/S_o) of Hydrocortisone acetate, Hydrocortisone caprylate and Progesterone as a function of the concentration of bile salt / lecithin micellar solution (3:1 ratio).



N.B. Symbols represent the mean of three replicates, error bars are standard error.

Figure 6.2 Solubility enhancement (S/S_o) of steroids in the presence of bile salt (15 mM) and bile salt / lecithin micellar solution (15 mM) as a function of log P_{HPLC}.



H = Hydrocortisone
HA = Hydrocortisone acetate
HB = Hydrocortisone butyrate
HC = Hydrocortisone caprylate
HV = Hydrocortisone valerate
E = Ethynylestradiol
P = Progesterone
T = Testosterone
TA = Testosterone acetate
TE = Testosterone enanthate

Figure 6.3 Log Solubilization ratio (log *SR*) in bile salt micellar solution (15 mM) as a function of log P_{HPLC}.

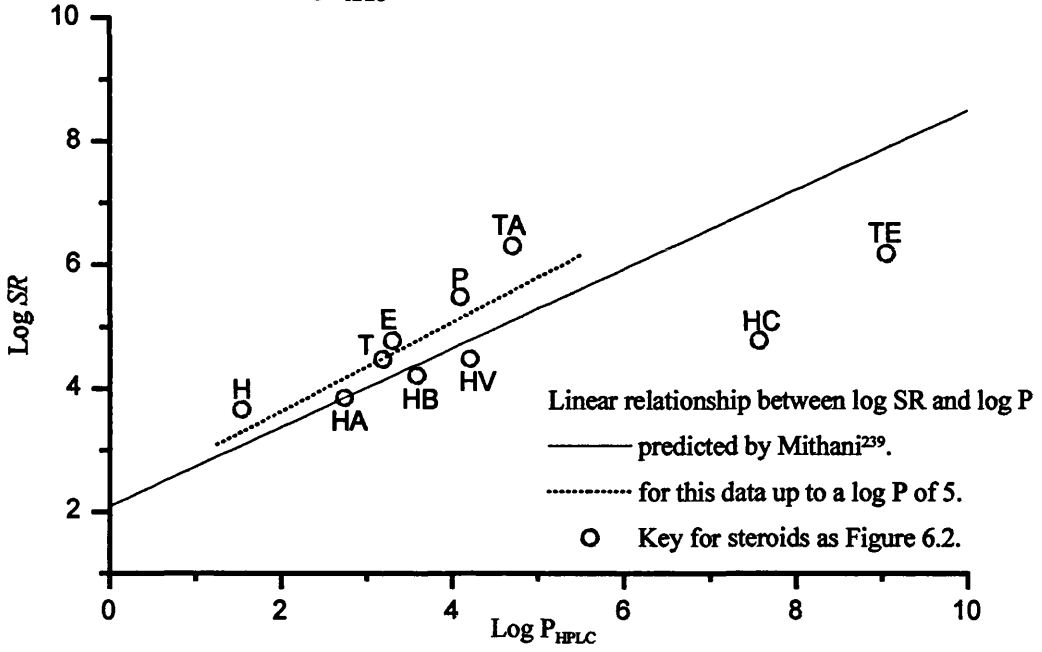


Figure 6.4 Solubility enhancement ratios (S/S_o) for steroids as a function of log P_{HPLC} to highlight behaviour of the Hydrocortisone esters.

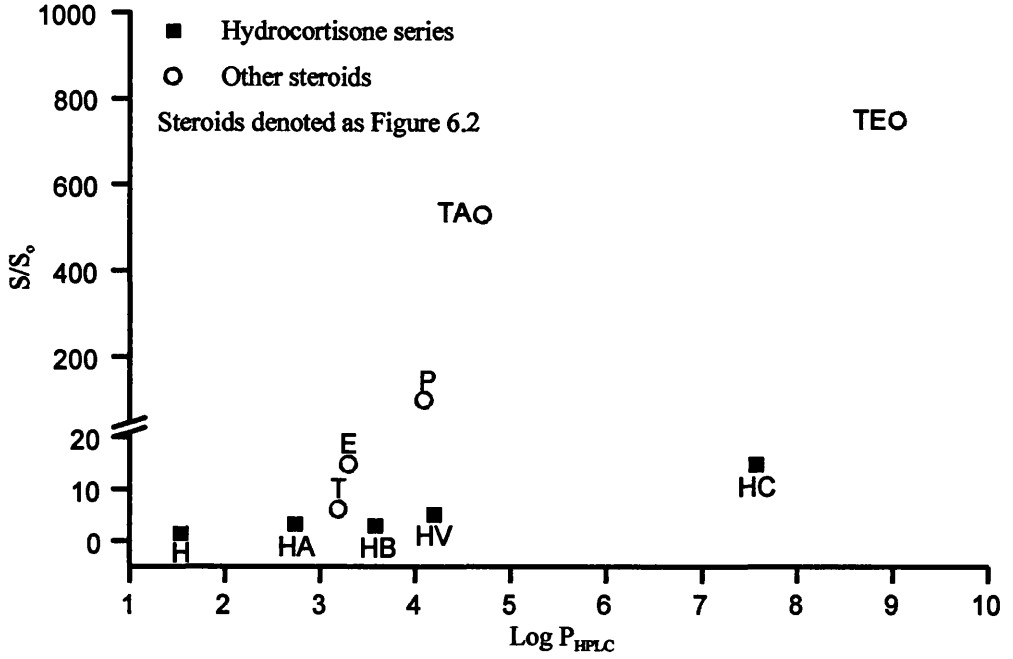


Figure 6.5 Influence of the bile salt to lecithin ratio of the micellar solution on the extent of solubility enhancement (S/S_o) for Progesterone.

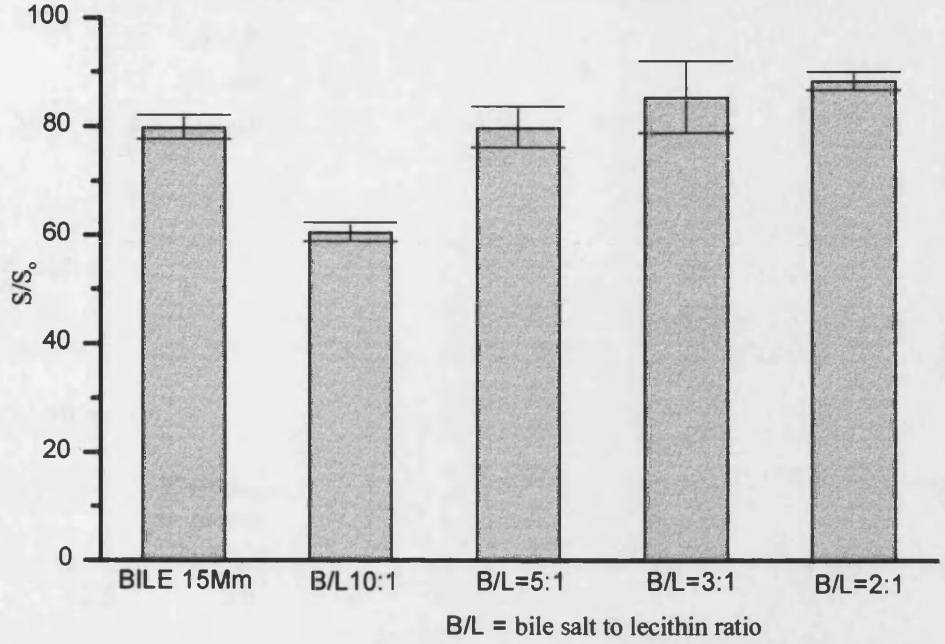


Figure 6.6 Influence of the bile salt to lecithin ratio of the micellar solution on the extent of solubility enhancement (S/S_o) for Testosterone acetate.

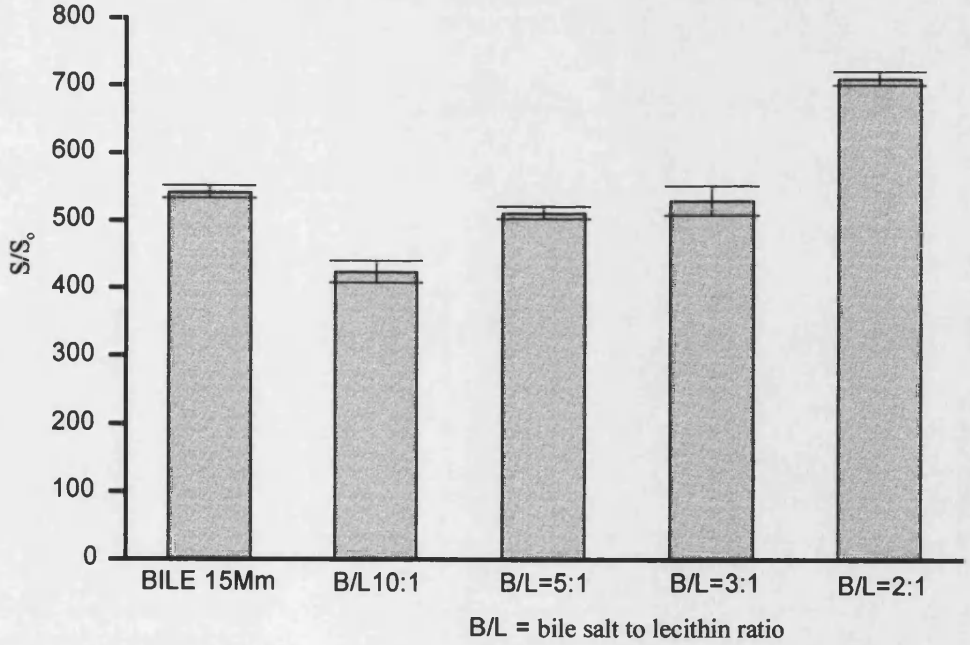


Figure 6.7 Influence of mixed lipid (fatty acid + monoglyceride) / bile salt micellar solution on the solubility enhancement (S/S_o) of steroids as a function of log P_{HPLC}.

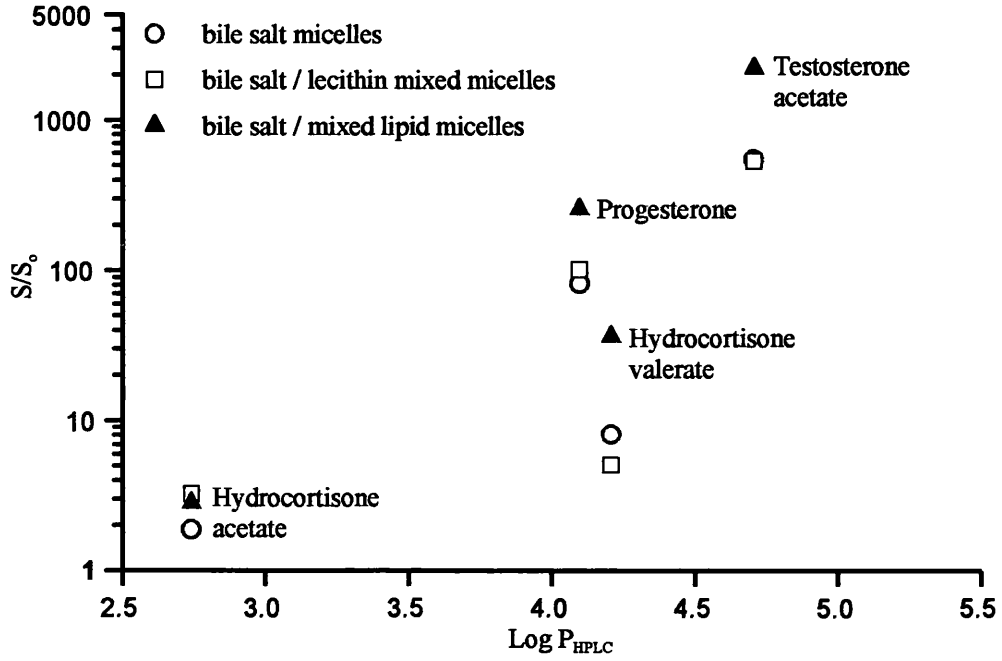


Figure 6.8a Dependence of log capacity factor (log k') on concentration of methanol in the mobile phase for steroids listed.

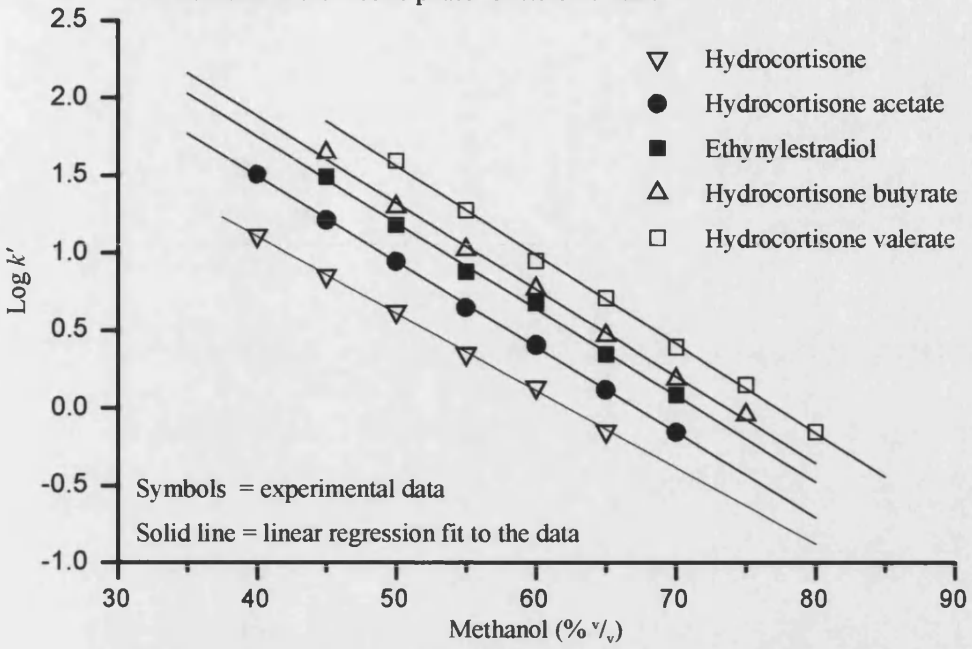


Figure 6.8b Dependence of log capacity factor (log k') on concentration of methanol in the mobile phase for steroids listed.

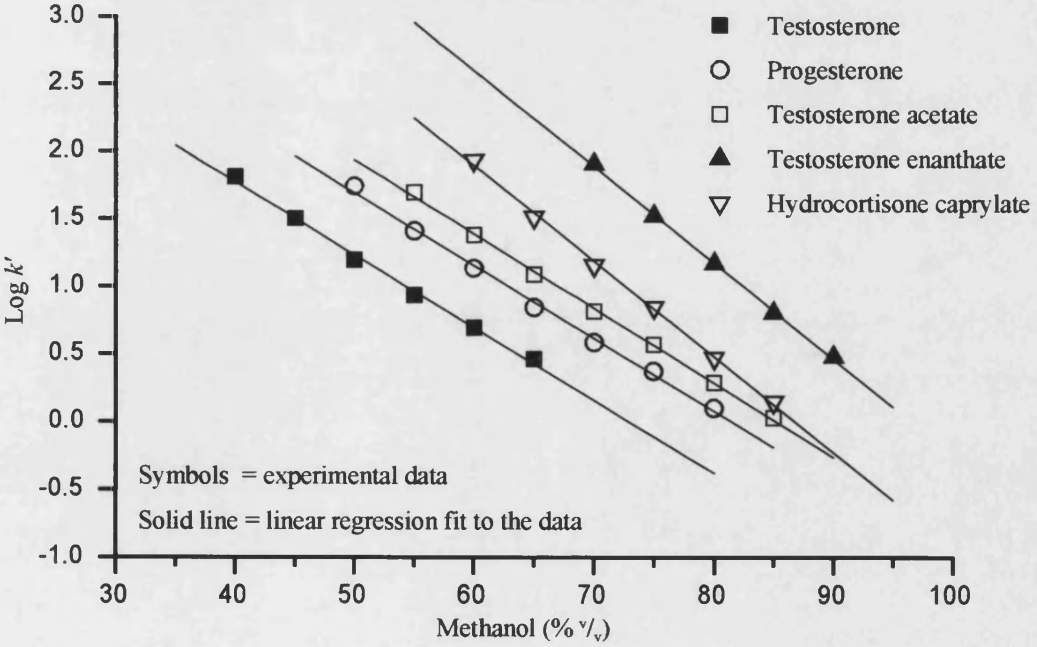
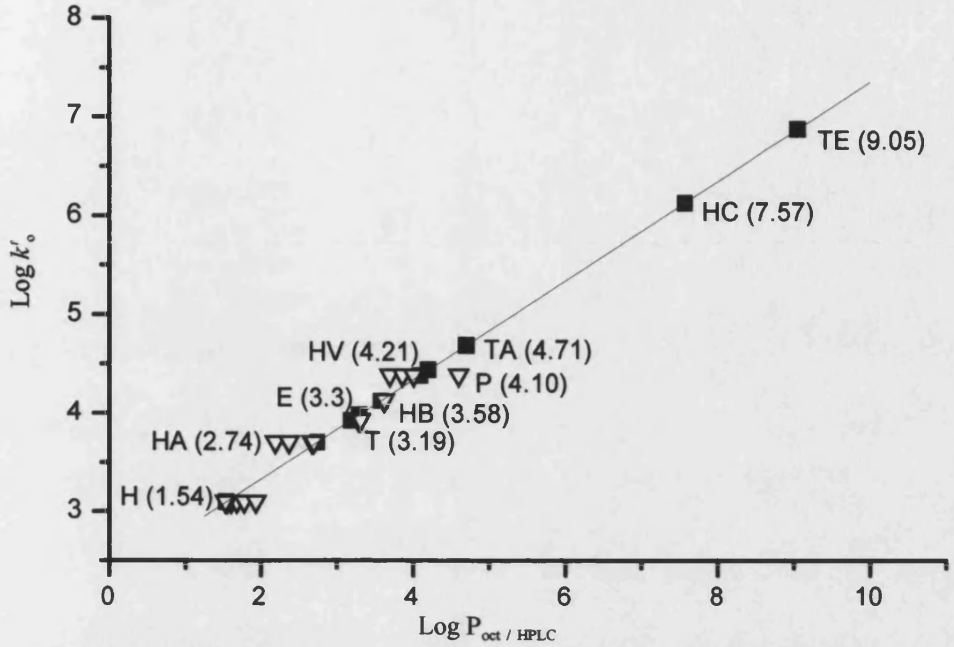


Figure 6.9 Calibration curve for determination of log P_{HPLC} values from log k'_o.



■ Experimentally determined log P_{HPLC} (actual values in parentheses).
▽ Literature log P_{oct} values used for construction of regression line (illustrated).
Steroids as denoted below:

H = Hydrocortisone
HA = Hydrocortisone acetate
HB = Hydrocortisone butyrate
HC = Hydrocortisone caprylate
HV = Hydrocortisone valerate
E = Ethynylestradiol
P = Progesterone
T = Testosterone
TA = Testosterone acetate
TE = Testosterone enanthate

Chapter 7 - A mathematical model for estimation of the fraction dose absorbed from an emulsion formulation.

7.1 Introduction

It is difficult to predict which drugs may benefit from formulation in a lipid based system and the literature gives sparse information on this. Recently the US Food and Drug Administration adopted the Biopharmaceutical Drug Classification scheme[278]. This scheme relates the rate and extent of drug absorption to the parameters of drug dissolution and permeability across the GI membrane. The drugs are classified according to the following descriptions into one of four classes:

Class	Solubility	Permeability
I	High	High
II	Low	High
III	High	Low
IV	Low	Low

Table 7.1 The Biopharmaceutic Drug Classes.

The majority of hydrophobic drugs would be expected to fall within Class II, as they easily cross the intestinal mucosa whilst exhibiting characteristics of low aqueous solubility. Dissolution into gastrointestinal fluid then becomes the rate limiting step of the absorption process as the rate at which drug dissolution occurs is too slow to enable all of the drug to dissolve within the transit time of the dosage form through the gut.

As most dosage forms for oral administration contain drug in the solid state, dissolution rate limitation can be a significant factor in the bioavailability of hydrophobic drugs. A modified version of the Noyes-Whitney equation (Equation 7.1)[240] can be used to reveal factors likely to influence drug dissolution.

$$DR = \frac{dX}{dt} = \frac{AD}{h} \cdot \left(C_s - \frac{X_d}{V} \right) \qquad \text{Equation 7.1}$$

where

DR = dissolution rate

A = surface area available for dissolution

D = diffusion coefficient of the drug

h = thickness of the boundary layer adjacent to the dissolving drug surface

C_s = saturation solubility of the drug

X_d = amount of dissolved drug

V = volume of dissolution media

The saturation solubility, C_s has a major influence on dissolution rate as the higher the concentration of drug in the boundary layer, the stronger the driving force for dissolution. If the aqueous solubility of a drug is less than 100 μg / ml or the volume of fluid required to dissolve a single dose of drug is greater than 1 litre C_s is likely to limit the rate of dissolution[240]. The size of the drug particles is also relevant to the rate of dissolution as shown by the term A in Equation 7.1, which indicates that the greater the surface area of the particle, the higher the dissolution rate.

Both C_s in the GI tract and A may be increased by selection of a lipid-based formulation for delivery of a hydrophobic drug. On the basis that lipid in the formulation stimulates the lipid digestion cascade, formation of high numbers of bile salt and lecithin micelles in the intestine would result. C_s may therefore be increased by means of solubilization of drug within the micelles. The amphipathic nature of the bile salts may also result in a greater effective particle surface area, A for dissolution through wetting effects.

Dissolution rate may be altered by other mechanisms upon transference of the GI tract from the fasted to the fed state[4]. Diffusivity, D of a drug when solubilized within a micellar structure in the fed state tends to decrease compared to that of a free drug molecule due to the larger size of the micelle. The presence of food usually increases viscosity of the GI fluid which in turn may decrease diffusivity and thus reduce dissolution rate of the drug. Variation in pH along the GI tract can change the dissolution rate of ionizable drugs, which exhibit different C_s values according to the pH of their environment. Whether this is advantageous to bioavailability would depend on the pK_a of the drug.

The overall effect on the dissolution rate of poorly water-soluble drugs through stimulation of the lipid digestion cascade, (whether by means of a formulation or food), has been observed from many *in vitro* studies to be specific to the particular drug under investigation. This suggests a combination of physicochemical properties of a drug to be relevant to dissolution. *In vivo* dissolution behaviour of a particular drug may also be affected by physiological factors which can vary greatly. The large number of variables which may influence dissolution complicates efforts to predict whether a particular drug will exhibit dissolution limited absorption. *In vitro* dissolution tests remain useful to provide a rough guideline.

Mathematical modeling is another approach which may be used to assess the rate-limiting processes in drug absorption. A model to correlate *in vitro* drug dissolution and absorption parameters with *in vivo* bioavailability would be extremely useful. This would allow a reduction in the number of *in vivo* studies required in humans, aid selection of suitable candidates during drug discovery and in the context of the work here, forward development of oral drug delivery strategies[279]. A comprehensive model to predict the bioavailability of a drug would require a prohibitively large number of variables due to the complexity of the GI tract. However when designing a model it is best to simplify the stages involved, which enables the parameters of a model to be examined in turn. This approach should reveal the main factors affecting the bioavailability of a particular drug in the model, with the assumption that this reflects the *in vivo* situation.

There have been many attempts (reviewed by Yu[280]) to develop a theoretical model to predict oral drug absorption in humans. The rate and extent of drug absorption depends to varying degrees upon the physicochemical characteristics of the drug in question, physiological factors and the choice of dosage form[280]. In general the models have a small number of parameters which describe the aqueous solubility, dose and membrane permeability of a drug[4]. Selection of a model for use depends upon the nature of the information required, which can range from general drug absorption trends through to the rate of drug absorption.

The models discussed throughout the remainder of this chapter are classified as steady-state models based on a mass balance approach. These models estimate the fraction of dose absorbed but provide no information regarding changes in the rate of

absorption with time. A relatively recent theoretical macroscopic mass balance approach[279] enabled estimation of the fraction of dose absorbed in humans, based on parameters of drug solubility and membrane permeability. As an extension of this theory a further mathematical model using a microscopic mass balance (MMB) approach has been developed[112]. This model is particularly relevant for poorly water-soluble drugs as it takes into account dissolution rate and membrane permeability using a limited number of parameters. The MMB approach has been used with some success to predict the fraction of dose absorbed from aqueous suspensions of poorly water-soluble drugs.

To examine the bioavailability of poorly water-soluble drugs from a lipid-based formulation the MMB approach has been adapted here to an emulsion system. The MMB model as developed by Oh et al[112] for aqueous suspensions is described in some detail below followed by the adaptations made in this work to enable prediction of the fraction of dose absorbed from lipid-based formulations.

7.2 The microscopic mass balance approach for aqueous suspensions of drug.

The suspension MMB model[112] considers the intestine to be a cylindrical tube through which a suspension of drug particles of uniform radii is passed. Absorption of drug occurs from the tube through the intestinal membrane to the blood upon drug dissolution. The tube is divided into slices with the fluid within each slice assumed to be of uniform composition (complete radial mixing). For each slice the model has values for the volume of suspension flowing in and out, (the rate of which remains constant), the rate at which the drug is dissolving and the rate of drug absorption through the intestinal membrane to the blood. As one progresses from slice to slice the concentration of drug passed from one to the next consecutive slice is reduced and the size of the drug particle decreased. The slices are presumed to be of infinitesimal width, hence there are an infinite number of them and the model reduces to the solution of two differential equations. Figure 7.1 illustrates in simplified terms the compartments involved in the model.

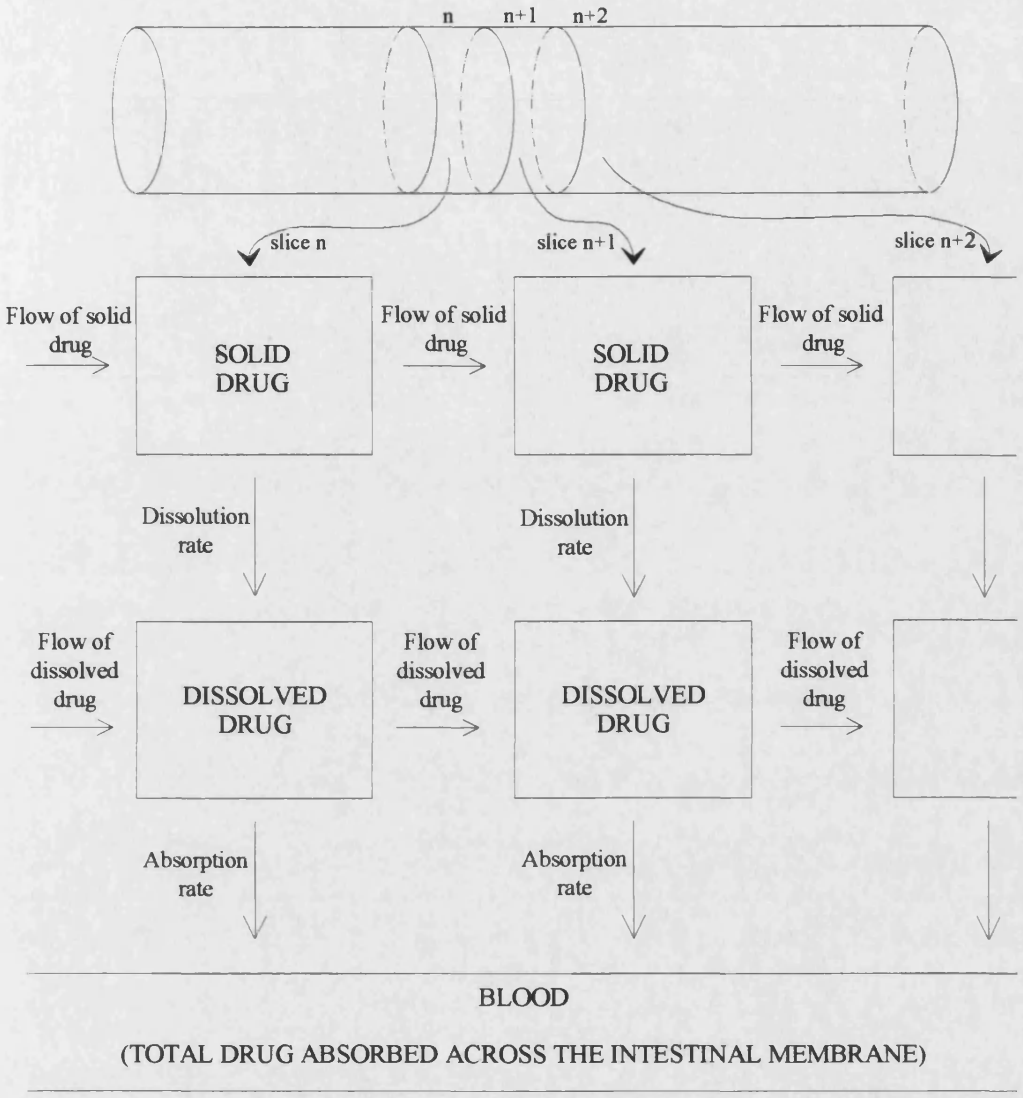


Figure 7.1 Flow diagram of the MMB approach[112] used to predict the fraction dose absorbed of suspensions of poorly water-soluble compounds.

Each column of compartments relates to a particular slice of which there are an infinite number. The first row of compartments represents the amount of drug in suspended particles in each slice, with the second row representing the amount of drug in solution in the intestinal fluid within the same slice. Arrows represent rates of drug motion between compartments - horizontal arrows represent flow associated with the physical flow of suspension along the intestine, whereas vertical arrows represent dissolution and absorption.

The model is expressed by the following two differential equations (Equations 7.2 / 7.3) as derived by Oh et al[112]:

$$\frac{dr^*}{dz^*} = -\frac{Dn}{3} \cdot \frac{1 - C^*}{r^*} \qquad \text{Equation 7.2}$$

$$\frac{dC^*}{dz^*} = Dn \cdot Do \cdot r^* \left(1 - C^*\right) - 2An \cdot C^* \qquad \text{Equation 7.3}$$

where the variables are defined as:

r^* = The particle radius as a fraction of the initial particle radius.

z^* = The position down the tube as a fraction of the total length of the tube.

C^* = The concentration of drug dissolved as a fraction of the aqueous solubility of the drug.

Only three dimensionless parameters are used to entirely characterize the efficiency of drug delivery in the model. These are the absorption number (An), the dose number (Do) and the dissolution number (Dn) defined below:

$$An = \frac{\text{radial absorption rate}}{\text{axial convection rate}}$$

$$Do = \frac{\text{dose concentration}}{\text{solubility}}$$

$$Dn = \frac{\text{residence time}}{\text{dissolution time}}$$

An

The absorption number is a measure of how efficiently drug is transported from the intestinal fluid into the blood, with the value of An increasing with the efficiency of the process. It includes terms which relate the ability of a drug to permeate the

intestinal membrane (radial absorption rate) with flow rate of the intestinal fluid (axial convection rate).

Do

The dose number describes the ability of a particular dose of drug to dissolve in the volume of intestinal fluid available. It includes terms for drug solubility, dose and volume of intestinal fluid, providing an element of pharmaceutical control. A small dose number will increase the fraction of the dose absorbed but manipulation of Do is restricted by consideration of the therapeutic efficacy of a drug. If a drug therefore requires a large dose and has low solubility characteristics Do will be high, reflecting a situation where the amount of drug administered in the dose is sufficient to produce non-sink conditions in the volume of intestinal fluid available.

Dn

The dissolution number is a measure of how efficiently drug is transported from the solid drug particles into the intestinal fluid, with the value of Dn increasing with the efficiency of the process. It includes terms for drug solubility, diffusivity, density and rate of change of the particle radius. The flow rate in the intestine is also included so that overall Dn relates the time taken for drug to dissolve (dissolution time) to the time the dosage form remains in the intestine (residence time). Dn is the main parameter of the model under pharmaceutical control, for example a reduction in the particle size of the drug will increase Dn. This reflects an improvement in the dissolution rate of a drug which may be relevant to the bioavailability of poorly water-soluble drugs.

From Equations 7.2 and 7.3 an expression can be derived for the total flow rate of drug through the intestinal membrane into the blood in terms of the parameters of the model. This total flow rate multiplied by the residence time (the same as that used to calculate Dn) equals the total amount of drug delivered. The efficiency of drug delivery can then be expressed as the fraction dose absorbed, F, given by the total amount of drug delivered divided by the dose. F is expressed in terms of the model parameters and variables by Equation 7.4.

$$F = 1 - \left\{r^*(1)\right\}^3 - \frac{C^*(1)}{Do} \qquad \text{Equation 7.4}$$

where

r^* (1) = The dimensionless radius of particles at the end of the intestine.

C^* (1) = The dimensionless concentration at the end of the intestine.

7.2.1 Conclusions of the suspension MMB model.

Hydrophobic drugs in Class II of the Biopharmaceutical Drug Classification scheme would be expected to have a low dissolution number in the suspension MMB model reflecting their poor aqueous solubility. In addition the nonpolar nature of the drugs will confer high membrane permeability characteristics resulting in a large absorption number[281]. In the case of these drugs the suspension MMB model shows that when dissolution number is small, F is low, hence only a small fraction of the dose will be absorbed. The value of F is not improved when the absorption number is increased for drugs with a low dissolution number. The extent of drug delivery is therefore dissolution rate limited with no influence from the rate of drug absorption across the intestinal membrane. This describes a situation where, as soon as the drug has dissolved in the intestinal fluid, absorption across the intestinal membrane into the blood occurs almost instantaneously. The model also shows that the dose number has very little influence unless it is greatly increased.

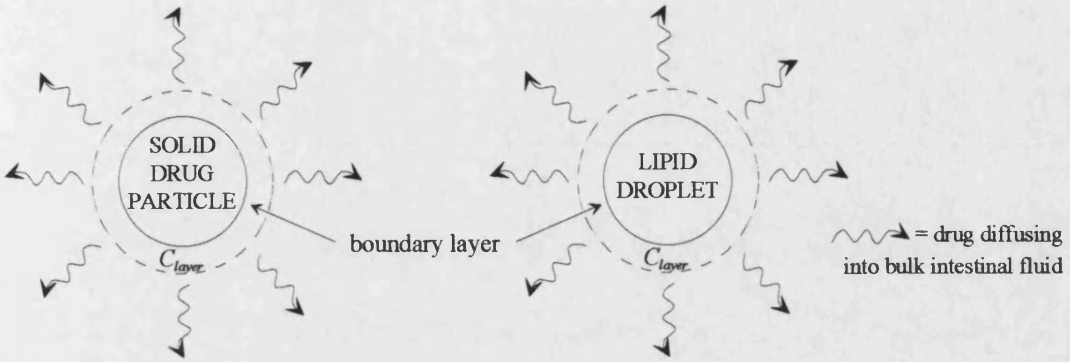
7.3 Adaptation of the microscopic mass balance approach for oil in water emulsions.

From the analysis provided by the suspension MMB model[112] it is apparent that if the flow of hydrophobic drug from the formulation into the intestinal fluid is increased, a higher fraction of the dose will be delivered. This may be achieved through the use of an alternative drug delivery method to enhance dissolution, such as a lipid-based formulation. The use of a lipid-based formulation for a Class II drug has been suggested throughout this thesis to be advantageous in avoiding dissolution by

various mechanisms. The majority of these mechanisms involved stimulation of the lipid digestion cascade, however simply administering the drug in a dissolved state within a lipidic solvent may be beneficial. This approach has potential to avoid the dissolution stage and enhance eventual drug bioavailability, irrespective of physiological lipid processing.

In the following discussion the MMB approach has been adapted in an attempt to model the fraction of dose delivered from an emulsion system. The aim was to examine the circumstances under which an emulsion system may give improved bioavailability compared to that from the same drug administered as an aqueous suspension. The adapted MMB model for emulsions (emulsion MMB model) represents a formulation where drug is in a dissolved state within lipid. Upon presentation of the formulation into the intestinal fluid the lipid disperses to form droplets of uniform radii. The lipid is regarded as non-digestible as the processes involved in lipid digestion are too complex to include within a predictive model. The major adaptation made to the suspension MMB model is based on the Noyes-Whitney equation[240] where dissolution is replaced in the emulsion MMB model by a partitioning process. For the suspension MMB model dissolution rate depends on drug dissolving into the boundary layer around a solid drug particle, from which drug diffusion into the bulk intestinal fluid occurs. In the case of the lipid droplets the rate of drug release depends on drug partitioning into the aqueous boundary layer around the droplet, followed by diffusion into the bulk intestinal fluid. Since the drug is partitioning across an interface between lipid and water, drug concentration in the lipid droplet, C_{lipid} and in the aqueous boundary layer around it, C_{layer} are related by the partition coefficient, P of the drug. The drug concentration in the boundary layer can therefore be calculated from P which enables the release of drug from the lipid droplet into the intestinal fluid to be described in exactly the same manner as for the suspension. An equation (Equation 7.5) analogous to the Noyes-Whitney equation was thus derived for the emulsion system; the reasoning behind the derivation is illustrated in Figure 7.2.

$$DR = \frac{dX}{dt} = \frac{AD}{h}\left(\frac{C_{lipid}}{P} - \frac{X_d}{V}\right) \qquad \text{Equation 7.5}$$



$$DR = \frac{dX}{dt} = \frac{AD}{h} \cdot \left(C_{layer} - \frac{X_d}{V} \right)$$

where C_{layer} = drug concentration in the aqueous boundary layer.

For solid drug particle

$$C_{layer} = C_s$$

$\therefore DR$ for the suspension ≡ the Noyes Whitney equation[240]

For lipid droplet

$$C_{layer} = \frac{P}{C_{lipid}}$$

where P = the partition coefficient

$\therefore DR$ for the emulsion = Equation 7.5.

Figure 7.2 The adaptation of the Noyes-Whitney equation for the emulsion MMB model.

Equation 7.5 was employed to derive the differential equations (Equations 7.6 / 7.7) analogous to Equations 7.2 / 7.3 using exactly the same MMB approach as Oh et al[112]. A full derivation of the emulsion MMB model is given in Appendix A.

$$\frac{dC^*_{lipid}}{dz^*} = -Df \cdot \left(\frac{C^*_{lipid}}{P} - C^* \right) \qquad \text{Equation 7.6}$$

$$\frac{dC^*}{dz^*} = Df.Vn.\left(\frac{C^*_{lipid}}{P} - C^* \right) - 2.An.C^* \qquad \text{Equation 7.7}$$

where the variables are defined as:

C^* = The concentration of drug in solution in the intestinal fluid as a fraction of the aqueous solubility of the drug (this takes the place of r^* for the suspension MMB approach).

C^*_{lipid} = The concentration of drug in the lipid droplets as a fraction of the aqueous solubility of the drug.

z^* = The position down the tube as a fraction of the total length of the tube.

In the same manner as for the suspension MMB model three parameters appear, the absorption number, An the volume number, Vn and the diffusion number, Df. The absorption number remains as described for the suspension MMB model with no change. The parameters of Vn and Df specific to the emulsion MMB model are defined below:

<u>The volume number</u>

$$Vn = \frac{\text{volume of lipid in dose}}{\text{luminal volume}}$$

The volume number is analogous to the dose number in the suspension MMB model and represents the amount of lipid in the dose within the model.

The diffusion number

$$Df = \frac{\text{residence time}}{\text{diffusion time}}$$

The diffusion number is analogous to the dissolution number in the suspension MMB model and represents the efficiency with which the drug diffuses from the boundary layer into the intestinal fluid.

In the same manner as for the suspension MMB model an equation can be derived for the fraction of dose adsorbed into the blood, F. This is given by Equation 7.8.

$$F = 1 - C^*_{lipid}(1) - \frac{C^*(1)}{Vn} \qquad \text{Equation 7.8}$$

where

$C^*_{lipid}(1)$ = The dimensionless concentration in the lipid droplets at the end of the intestine.

$C^*(1)$ = The dimensionless concentration in the intestinal fluid at the end of the intestine.

The solution of these equations is simpler than for the suspension MMB model, and can be solved analytically. This gives the expression for F in Equation 7.9.

$$F = 1 - \exp\left(-2 \cdot \frac{An}{1 + Dv \cdot P + \frac{2 \cdot An \cdot P}{Dd}}\right) \qquad \text{Equation 7.9}$$

where P = the partition coefficient of the drug.

7.3.1 Results from the emulsion MMB model.

Initially the model was employed to examine how the hydrophobicity of a drug may alter the fraction of dose absorbed, F from an emulsion system. In addition the effect of lipid droplet size on F was also examined to determine if the quality of the emulsion formed by a lipid formulation would influence the bioavailability of a drug. The hydrophobicity of the drug was represented by the log of the partition coefficient, $\log P$ which was increased from 0 to 10. The quality of the emulsion was defined by the radius of the lipid droplets, which varied across a range of 1 to 1000 μm. Other parameters required were taken from the suspension MMB model[112], where appropriate, and the drug was assumed to be delivered in 1 ml of lipid. F was then calculated using these values and an An of 10 and the results are illustrated in Figures 7.3 and 7.4.

Figure 7.3 shows the variation of F with lipid droplet radius for $\log P$ values of 1, 2, 3, 4 and 5. It is apparent from this figure that as the lipid droplet radius is decreased, F increases from 0 to a constant saturated value. When $\log P$ is high (> 5) the model shows that no drug is delivered, for drugs with a low $\log P$ (< 3) a saturated value of 1 is shown (representing complete delivery) and between these values the extent of delivery depends on $\log P$. The most important conclusion to be drawn from Figure 7.3 is that the efficiency of drug delivery, represented by F, is unchanged if the radius of the lipid droplet is decreased below 50 μm.
The same results are illustrated in Figure 7.4 from a different view point. F is shown as a function of $\log P$ for lipid droplet radii of 1, 10, 100 and 1000 μm. This perspective improves the visualization of $\log P$ dependence on lipid droplet size in terms of the efficiency of drug delivery.
Manipulation of the model can alter the above results. The lipid droplet radius below which no further increase in F is observed can be increased above the present value of 50 μm by increasing the residence time in the gut or by using a higher diffusivity. The fraction of dose delivered by high $\log P$ drugs can also be increased to a certain extent by increasing An or decreasing the volume number. In physical terms this

corresponds to increasing membrane permeability of the drug and increasing the concentration of drug in the lipid droplets respectively.

An absorption number of 10 was used for the above calculations, as this value had been suggested by Amidon et al[278] to represent drugs with the property of high membrane permeability. Since An is an estimated quantity it was desirable to assess the sensitivity of the model and the conclusions drawn from it with respect to variations in An. A confidence in An of 50 % has been described[280] therefore F was calculated using absorption numbers of 5, 10 and 15 (Figure 7.5) for a lipid droplet radius of 10 μm. Figure 7.5 shows that conclusions drawn from Figures 7.3 and 7.4 are not altered by An, although the maximum log P for efficient drug delivery tends to decrease slightly with decreasing An.

As emulsification of lipid in the stomach has been found to result in lipid droplets with radii in the range of 0.5 to 50 μm[17], it is reasonable to presume droplets in the same region could be achieved *in vivo* from a lipid-based formulation. By assuming a lipid droplet radius of less than 50 μm F is effectively independent of the droplet radius and diffusivity. In view of this Equation 7.10 can be obtained (by taking an infinitely small r).

$$F = 1 - \exp\left(-2 \cdot \frac{An}{1 + Vn \cdot P}\right) \qquad \text{Equation 7.10}$$

Therefore in order to calculate the fraction dose absorbed within the emulsion MMB model using Equation 7.10 the only parameters required are the partition coefficient and An of the drug and the volume number, Vn for the dose.

7.4 Can an emulsion system improve bioavailability of hydrophobic drugs over that given by an aqueous suspension ?

The emulsion and suspension MMB models enable prediction of the bioavailability of poorly water-soluble drugs when dosed as an emulsion or as a suspension respectively. To determine if an emulsion may improve the bioavailability of

hydrophobic drugs compared to the use of an aqueous suspension, ten steroidal compounds (listed in section 6.2) with varying degrees of hydrophobicity were selected. Certain parameters required by the models, namely C_s (represented by solubility in aqueous buffer solution) and P (represented by P_{HPLC}) had already been determined experimentally in Chapter 6 for these drugs.

The emulsion MMB model (Equation 7.10) was employed to calculate F for each steroid using parameters of P_{HPLC}, an An of 10 (as used by Amidon et al[278]), a luminal volume of 250 ml and 1 ml of lipid to deliver the drug. The resulting calculated values of F are shown in Table 7.2 as percent dose absorbed.

For the same steroids Equation 7.4 was applied to evaluate F from an aqueous suspension. An was again taken to be 10, whereas Dn and Do for the drugs were evaluated using the same parameters as for digoxin by Amidon et al[278], with appropriate adjustments made for a dose of 50 mg and differences in C_s. These values enabled calculation of F, which is expressed in Table 7.2 as percent dose absorbed.

Drug	Log P_{HPLC}	% dose absorbed	
		Suspension MMB	Emulsion MMB
Hydrocortisone	1.54	100.0	100.0
Hydrocortisone acetate	2.74	9.8	99.8
Testosterone	3.19	14.3	93.8
Ethynylestradiol	3.30	12.8	89.2
Hydrocortisone butyrate	3.58	65.3	70.8
Progesterone	4.10	1.1	32.3
Hydrocortisone valerate	4.21	19.7	26.2
Testosterone acetate	4.71	0.2	9.3
Hydrocortisone caprylate	7.57	2.2	0.01
Testosterone enanthate	9.05	1.5	0.0004

Table 7.2 Percent dose absorbed calculated using a MMB approach for a range of steroidal compounds when delivered as a suspension or an emulsion.

Table 7.2 illustrates the predicted percentage of a 50 mg dose of steroid likely to be absorbed when using different delivery systems. Results from the suspension MMB model could be taken to represent a disintegrated tablet and reflect the influence of the aqueous solubility of a drug on efficiency of delivery. The emulsion MMB model reveals the influence of the hydrophobicity of a drug, represented by log P, on the

percentage of dose absorbed from lipid droplets. These droplets could be presumed to result from dispersion of a lipid-based formulation in intestinal fluid.

The results show that the delivery of drug is more efficient from the emulsion model for drugs with a log P in the range of 1.5 to 5. Both models delivered 100 % of the least hydrophobic drug tested (hydrocortisone, log P 1.54) which is probably due to the higher aqueous solubility of this drug. For a log $P > 5$ the suspension model showed minimal delivery whereas the emulsion model failed to deliver any drug. Lack of delivery for highly hydrophobic drugs from the emulsion model is probably unrealistic since the model does not take into account the process of lipid digestion, which is likely to improve drug delivery. The emulsion model therefore indicates the importance of digestion for absorption of drugs with high hydrophobicity, which may otherwise remain partitioned in lipid until excretion.

Overall the models suggest these steroids would benefit from delivery in an emulsion system as the predicted bioavailability is superior to that from a suspension (by a factor of up to 46, but typically 10) provided log P is less than 5. For drugs with a higher log P digestion of lipid gives further potential to increase the efficiency of delivery from an emulsion, whereas delivery from a suspension would remain severely limited by the low aqueous solubility of the drugs.

Figure 7.3 Variation of fraction dose absorbed, F with lipid droplet radius, r for log P values indicated.

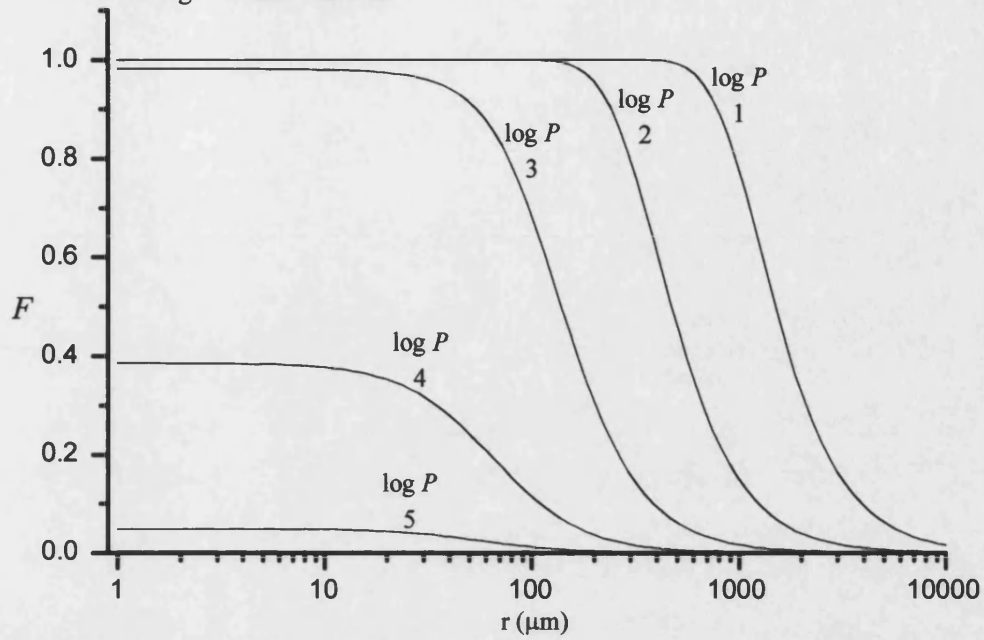


Figure 7.4 Variation of fraction dose absorbed, F as a function of log P for lipid droplet radii, r indicated.

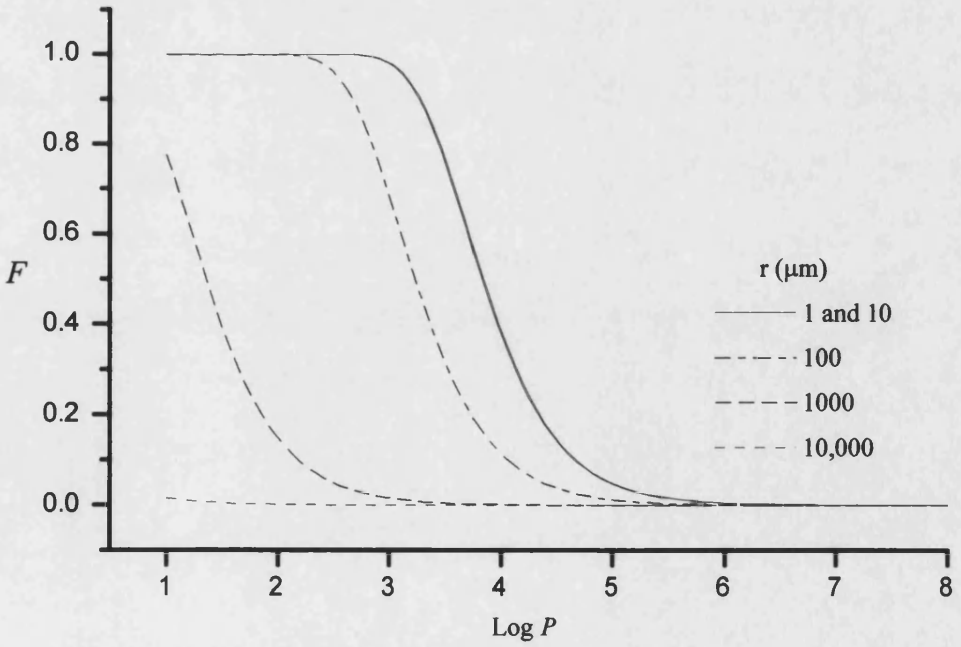
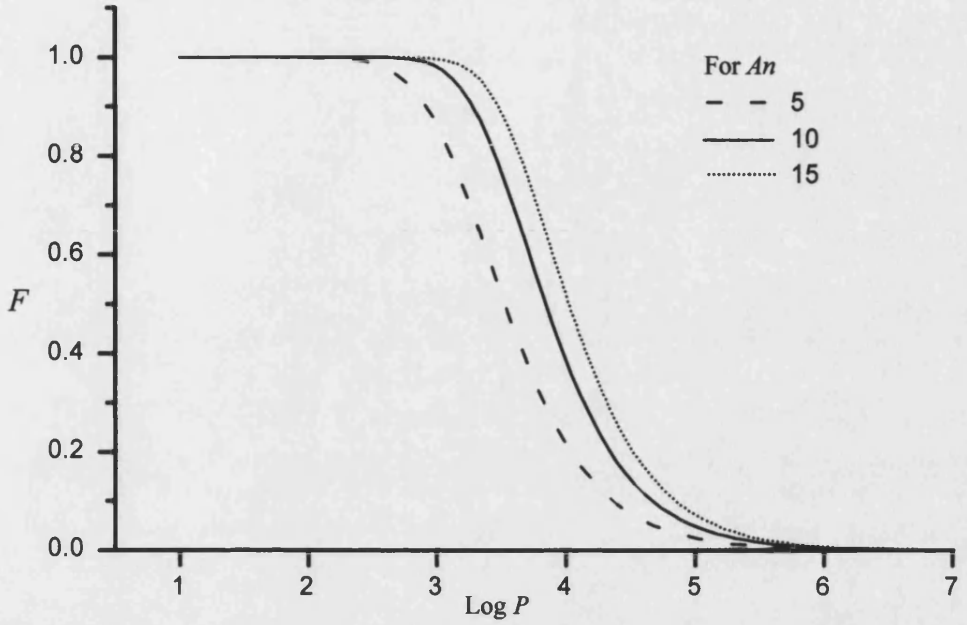


Figure 7.5 Variation of fraction dose absorbed, F as a function of log P for absorption numbers, An indicated.



Chapter 8 - Conclusions

The work performed in this thesis has provided additional information regarding the use of lipid-based formulations for drugs which exhibit dissolution limited absorption. As physiological lipid processing has been suggested to enhance bioavailability from a lipid-based formulation[1], the possible rate of digestion of lipid-based formulations in the duodenum was assessed using an *in vitro* model of lipolysis. This model enabled examination of the rate at which a lipid substrate was digested by lipase, in the presence of non-ionic surfactants likely to be included within a lipid-based formulation.

The results indicated that hydrophilic surfactants with hydrophile / lipophile balance (HLB) values in the range of 13 to 15 were, in general, potent inhibitors of lipase activity towards medium chain triglyceride. Surfactants with dominant lipophilic character were also able to inhibit lipase activity although the inhibition was less severe, with limited lipolysis able to occur from the start of the assay. When both hydrophilic and lipophilic surfactants were present in the system a further phenomenon was observed, where the lipophilic surfactant appeared able to relieve inhibition due to the hydrophilic surfactant.

A theory to explain the effect of non-ionic surfactants on the activity of lipase is proposed. Hydrophilic surfactants, orientated at the lipid / water interface may form a hydrophilic barrier around the substrate by means of their ethoxy chains. Initially this barrier would prevent access of colipase to the triglyceride interface, and thus inhibit lipase activity. However, eventually some colipase would be expected to bind followed by lipase, with the consequent production of lipolytic products. Accumulation of these products at the interface may act to promote partitioning of colipase into the substrate surface, with a resultant increase in lipase activity and reduction in the extent of inhibition from the surfactant molecules. Lipophilic surfactants when present in the system are suggested to speed this process up, either by containing lipolytic products as part of their composition or by providing an additional source of substrate for lipase.

In any event inhibition caused by surfactants examined in this work is eventually overcome and hence is concluded to be of a reversible nature. This is important when relating the results from the *in vitro* model to a situation which may occur upon administration of a lipid-based formulation *in vivo*. A formulation which is initially unavailable to lipase activity *in vitro* will probably not remain in this state for long *in vivo*. The mechanisms of emulsification in the gut will disperse the formulation, and probably alter the nature of the interface between the GI fluid and the surface of the formulation droplets to such an extent that the inhibitory layer of hydrophilic surfactant is disrupted. Lipase present in the duodenum will thus be able to digest lipid in the formulation, with any remaining inhibition from the surfactant rapidly overcome. This proposal is based on the assumption that the concentration of lipid present within the formulation is sufficient to stimulate the 'lipid digestion cascade' and thus lipase secretion. If not, digestion of the formulation is unlikely, with the drug possibly remaining partitioned in the lipid until excretion. Administration of the formulation in the fed state would increase the likelihood of stimulation of the lipid digestion cascade, however this may introduce complications with regards to patient compliance and variation in the nature of the food eaten.

Alternatively, to bypass any requirement for stimulation of the lipid digestion cascade, the drug could be administered in a dissolved state within a self-emulsifying drug delivery system (SEDDS). The excellent dispersion of these formulations upon release into the aqueous GI fluid may alone be sufficient to improve drug absorption. The drug would partition from the fine dispersion of droplets formed by the SEDDS and be held in a dissolved state in the solubilizing phase formed by components of the formulation ready for absorption. The efficiency of the partitioning process of hydrophobic drugs from lipid droplets into the aqueous fluid has been predicted by the emulsion microscopic mass balance (MMB) model (see Chapter 7). This model suggests that partitioning of drug from a lipid droplet may be a promising method to improve the bioavailability of drugs with a log $P < 5$, over that achieved from an aqueous suspension of drug. Problems may thus be encountered with SEDDS if the drug is too hydrophobic to partition from the droplets formed by dispersion of the

formulation. An additional complication may be introduced if the drug has a tendency to precipitate upon release into an aqueous environment.

A possible technique which may enable stimulation of the lipid digestion cascade without the use of a high lipid volume could involve the formulation of a SEDDS which includes as part of its composition lipolytic digestion products. These may be incorporated in the SEDDS by using surfactants for the formulation which contain lipolytic products as part of their make-up.

The limited concentration of lipolytic products formed by gastric lipase activity on ingested lipid have been stated to promote immediate release of pancreatic lipase upon entry into the duodenum[16]. Based on this administration of a SEDDS which forms a well dispersed solubilizing phase containing lipolytic products in the fasted state could mimic the fed state. The lipolytic products may stimulate secretion of bile and lipolytic enzymes from the gallbladder and pancreas respectively, thus bypassing any requirement for initial digestion of lipid.

The advantages of the lipid digestion cascade upon the bioavailability of poorly water-soluble drugs should then come into play. The partitioning of highly hydrophobic drugs from droplets of the formulation is likely to be enhanced by digestion. The resulting higher concentration of bile salt and lecithin micelles in the GI fluid will also raise the solubilizing capacity of the dispersion formed by the SEDDS for the drug. The solubilizing capacity of bile salt and lecithin micelles for hydrophobic drugs (demonstrated in Chapter 6) may reduce the chance of precipitation of drug in the aqueous fluid and aid transport of drug in the micelles to the brush border membrane for absorption.

In terms of the suitability of a drug for formulation within a lipid-based system the log P of a drug provides a useful guideline. In general drug is required to be soluble within the lipid-based formulation, this has been assumed in the emulsion MMB model although it is by no means always the case. Manipulation of the formulation in terms of co-solvents and surfactants is sometimes required. The improved drug bioavailability noted from lipid-based solid dispersions[1], where drug is present in excess of its solubility in a lipid vehicle, show that it may not be necessary to

originally have the drug in a dissolved form; the presence of material to improve solubilization of the drug may be sufficient.

In summary lipid-based formulations can be considered to have potential to improve the bioavailability of poorly water-soluble drugs compared to that achieved from a solid dosage form. The major advantage being the ability to present the drug to the gut in a dissolved form thus enabling avoidance of the dissolution stage which is the rate limiting step to the absorption of these drugs. Further work investigating lipid-based formulations and their effect upon bioavailability is required to clarify the requirements of a lipid-based formulation. Comparison of the bioavailability from SEDDS containing lipolytic products, and those which do not, would be of interest.

References

1 Humberstone, A.J. and Charman, W.N. (1997) Lipid-based vehicles for the oral delivery of poorly water soluble drugs. *Adv. Drug Delivery Rev.* **25**, 103-128.

2 Crounse, R.G. (1961) Human pharmacology of griseofulvin: The effect of fat intake on gastrointestinal absorption. *J. Invest. Dermatol.* **37**, 529-533.

3 Crounse, R.G. (1963) Effective use of griseofulvin. *Arch. Dermatol.* **87**, 176-178.

4 Charman, W.N., Porter, C.J.H., Mithani, S. and Dressman, J.B. (1997) Physicochemical and Physiological Mechanisms for the Effects of Food on Drug Absorption: The Role of Lipids and pH. *J. Pharm. Sci.* **86**, 269-282.

5 Welling, P.G. (1989) The effects of food on drug absorption. *Pharmacol. Ther.* **43**, 425-444.

6 Thomson, A.B.R., Schoeller, C., Keelan, M., Smith, L. and Clandinin, M.T. (1993) Lipid absorption: passing through the unstirred layers, brush-border membrane, and beyond. *Can. J. Physiol. Pharmacol.* **71**, 531-555.

7 Tso, P. (1985) Gastrointestinal Digestion and Absorption of Lipid. *Adv. Lipid. Res.* **21**, 143-186.

8 Carey, M.C., Small, D.M. and Bliss, C.M. (1983) Lipid Digestion and Absorption. *Ann. Rev. Physiol.* **45**, 651-677.

9 Moreau, H., Bernadac, A., Gargouri, Y., Benkouka, F., Laugier, R. and Verger, R. (1989) Immunocytolocalization of human gastric lipase in chief cells of the fundic mucosa. *Histochemistry* **91**, 419-423.

10 Moreau, H., Laugier, R., Gargouri, Y., Ferrato, F. and Verger, R. (1988) Human preduodenal lipase is entirely of gastric fundic origin. *Gastroenterology* **95**, 1221-1226.

11 Tiruppathi, C. and Balasubramanian, K.A. (1982) Purification and properties of an acid lipase from human gastric juice. *Biochim. Biophys. Acta.* **712**, 692-697.

12 Gargouri, Y., Piéroni, G., Riviére, C., Sauniére, J.F., Sarda, L. and Verger, R. (1986) Kinetic assay of human gastric lipase on short- and long-chain triacylglycerol emulsions. *Gastroenterology* **91**, 919-925.

13 Hamosh, M. (1984) Lingual lipase. In: B. Borgström and H.L. Brockman (Eds.), Lipases. Elsevier, Amsterdam, pp. 49-81.

14 Gargouri, Y., Piéroni, G., Lowe, P.A., Sarda, L. and Verger, R. (1986) Human gastric lipase: The effect of amphiphiles. *Eur. J. Biochem.* **156**, 305-310.

15 Gargouri, Y., Julien, R., Piéroni, G., Verger, R. and Sarda, L. (1984) Studies on the inhibition of pancreatic and microbial lipases by soybean proteins. *J. Lipid. Res.* **25**, 1214-1221.

16 Gargouri, Y., Piéroni, G., Riviére, C., Lowe, P.A., Sauniére, J.F., Sarda, L. and Verger, R. (1986) Importance of human gastric lipase for intestinal lipolysis: an *in vitro* study. *Biochim. Biophys. Acta.* **879**, 419-423.

17 Armand, M., Borel, P., Dubois, C., Senft, M., Peyrot, J., Salducci, J., Lafont, H. and Lairon, D. (1994) Characterization of emulsions and lipolysis of dietary lipids in the human stomach. *Am. J. Physiol.* **266**, G372-G381.

18 Dutta, S.K., Hamosh, M., Abrams, C.K., Hamosh, P. and Hubbard, V.S. (1982) Quantitative estimation of lingual lipase activity in the upper small intestine in adult patients with pancreatic insufficiency. *Gastroenterology* **82**, 1047 (Abstr.).

19 Sarda, L. and Desnuelle, P. (1958) Action de la lipase pancréatique sur les esters en émulsion. *Biochim. Biophys. Acta* **30**, 513-521.

20 Borgström, B. and Erlanson-Albertsson, C. (1984) Pancreatic colipase. In: B. Borgström and H.L. Brockman (Eds.), Lipases. Elsevier, Amsterdam, pp. 151-183.

21 Patton, J.S., Albertsson, P.A., Erlanson, C. and Borgström, B. (1978) Binding of porcine pancreatic lipase and colipase in the absence of substrate studied by two-phase partition and affinity chromatography. *J. Biol. Chem.* **253**, 4195-4202.

22 Stadler, P., Kovac, A., Haalck, L., Spener, F. and Paltauf, F. (1995) Stereoselectivity of microbial lipases: The substitution at position *sn*-2 of triacylglycerol analogs influences the stereoselectivity of different microbial lipases. *Eur. J. Biochem.* **227**, 335-343.

23 Larsson, A. and Erlanson-Albertsson, C. (1981) The identity and properties of two forms of activated colipase from porcine pancreas. *Biochim. Biophys. Acta* **664**, 538-548.

24 Erlanson-Albertsson, C. (1992) Pancreatic colipase. Structural and physiological aspects. *Biochim. Biophys. Acta.* **1125**, 1-7.

25 Larsson, A. and Erlanson-Albertsson, C. (1991) The effect of pancreatic procolipase and colipase on pancreatic lipase activation. *Biochim. Biophys. Acta* **1083**, 283-288.

26 Winkler, F.K., D'Arcy, A. and Hunziker, W. (1990) Structure of human pancreatic lipase. *Nature.* **343**, 771-774.

27 Ollis, D.L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S.M., Harel, M., Remington, S.J., Silman, I., Schrag, J., Sussman, J.L., Verschueren, K.H.G. and Goldman, A. (1992) The α/β hydrolase fold. *Prot. Eng.* **5**, 197-211.

28 van Tilbeurgh, H., Sarda, L., Verger, R. and Cambillau, C. (1992) Structure of the pancreatic lipase-procolipase complex. *Nature.* **359**, 159-162.

29 Rugani, N., Carrière, F., Thim, L., Borgstrom, B. and Sarda, L. (1995) Lipid binding and activating properties of porcine pancreatic colipase split at the Ile^{79}-Thr^{80} bond. *Biochim. Biophys. Acta.* **1247**, 185-194.

30 Lüthi-Peng, Q., Märki, H.P. and Hadváry, P. (1992) Identification of the active-site serine in human pancreatic lipase by chemical modification with tetrahydrolipstatin. *FEBS Lett.* **299**, 111-115.

31 Brzozowski, A.M., Derewenda, U., Derewenda, Z.S., Dodson, G.G., Lawson, D.M., Turkenburg, J.P., Bjorkling, F., Huge-Jensen, B., Patkar, S.A. and Thim, L. (1991) A model for interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex. *Nature* **351**, 491-494.

32 van Tilbeurgh, H., Egloff, M., Martinez, C., Rugani, N., Verger, R. and Cambillau, C. (1993) Interfacial activation of the lipase-procolipase complex by mixed micelles revealed by X-ray crystallography. *Nature* **362**, 814-820.

33 Jennens, M.L. and Lowe, M.E. (1994) A surface loop covering the active site of human pancreatic lipase influences interfacial activation and lipid binding. *J. Biol. Chem.* **269**, 25470-25474.

34 Aoubala, M., de La Fournière, L., Douchet, I., Abousalham, A., Daniel, C., Hirn, M., Gargouri, Y., Verger, R. and De Caro, A. (1995) Human pancreatic lipase: Importance of the hinge region between the two domains, as revealed by monoclonal antibodies. *J. Biol. Chem.* **270**, 3932-3937.

35 Egloff, M., Marguet, F., Buono, G., Verger, R., Cambillau, C. and van Tilbeurgh, H. (1995) The 2.46 Å resolution structure of the pancreatic lipase-colipase complex inhibited by a C11 alkyl phosphonate. *Biochemistry* **34**, 2751-2762.

36 Lowe, M.E. (1994) Pancreatic triglyceride lipase and colipase: Insights into dietary fat digestion. *Gastroenterology* **107**, 1524-1536.

37 Embleton, J.K. and Pouton, C.W. (1997) Structure and function of gastro-intestinal lipases. *Adv. Drug Deliv. Rev.* **25**, 15-32.

38 Armand, M., Borel, P., Pasquier, B., Dubois, C., Senft, M., Andre, M., Peyrot, J., Salducci, J. and Lairon, D. (1996) Physicochemical characteristics of emulsions during fat digestion in human stomach and duodenum. *Am. J. Physiol.* **34**, G172-G183.

39 Staggers, J.E., Hernell, O., Stafford, R.J. and Carey, M.C. (1990) Physical-chemical behavior of dietary and biliary lipids during intestinal digestion and absorption. 1. Phase behavior and aggregation states of model lipid systems patterned after aqueous duodenal contents of healthy adult human beings. *Biochemistry* **29**, 2028-2040.

40 Hernell, O., Staggers, J.E. and Carey, M.C. (1990) Physical-chemical behavior of dietary and biliary lipids during intestinal digestion and absorption. 2. Phase analysis and aggregation states of luminal lipids during duodenal fat digestion in healthy adult human beings. *Biochemistry* **29**, 2041-2056.

41 Sémériva, M., Dufour, C. and Desnuelle, P. (1971) On the probable involvement of a histidine residue in the active site of pancreatic lipase. *Biochemistry* **10**, 2143-2149.

42 Westergaard, H. and Dietschy, J.M. (1974) Delineation of the dimensions and permeability characteristics of the two major diffusion barriers to passive mucosal uptake in the rabbit intestine. *J. Clin. Invest.* **174**, 718-732.

43 Narayanan, V.S. and Storch, J. (1996) Fatty-acid transfer in taurodeoxycholate mixed micelles. *Biochemistry* **35**, 7466-7473.

44 Westergaard, H. and Dietschy, J.M. (1976) The mechanism whereby bile acid micelles increase the rate of fatty acid and cholesterol uptake into the intestinal mucosal cell. *J. Clin. Invest.* **58**, 97-108.

45 Thomson, A.B.R., Schoeller, C., Keelan, M., Smith, L. and Clandinin, M.T. (1993) Lipid absorption: passing through the unstirred layers, brush-border membrane, and beyond. *Can. J. Physiol. Pharmacol.* **71**, 531-555.

46 Levitt, M.D., Strocchi, A. and Levitt, D.G. (1992) Human jejunal unstirred layer: evidence for extremely efficient luminal stirring. *Am. J. Physiol.* **262**, G593-G596.

47 Blair, J.A., Lucas, M.L. and Matty, A.J. (1975) Acidification in the rat proximal jejunum. *J. Physiol.* (London) **245**, 333-350.

48 Lucas, M.L. (1984) Pharmacology of intestinal permeation. II. T.Z. Csaky (Ed.), Springer-Verlag, Tokyo, pp.119-163.

49 Shiau, Y.-F. (1990) Mechanism of intestinal fatty acid uptake in the rat: the role of an acidic microclimate. *J. Physiol.* (London) **421**, 463-474.

50 Shiau, Y.-F. and Levine, G.M. (1980) pH dependence of micellar diffusion and dissociation. *Am. J. Physiol.* **239**, G177-G182.

51 Patton, J.S. (1986) Is the intestinal membrane bilayer freely permeable to lipophilic molecules ? In: F. Alverado and C.H. Van Os (Eds.), INSERM symposium No.26. Elsevier Science Publishers, pp.33-36.

52 Stremmel, W., Lotz, G., Strohmeyer, G. and Berk, P.D. (1985) Identification, isolation and partial characterization of a fatty acid binding protein from rat jejunal microvillus membranes. *J. Clin. Invest.* **75**, 1068-1076.

53 Clark, S.D. and Armstrong, M.K. (1989) Cellular lipid binding proteins: expression, function and nutritional regulation. *FASEB J.* **3**, 2480-2487.

54 Nunn, W.D., Colburn, R.W. and Black, P.N. (1986) Transport of long-chain fatty acids in *Escherichia coli*. *J. Biol. Chem.* **261**, 167-171.

55 Lewis, M.C. and Root, C. (1990) *In vivo* transport kinetics and distribution of taurocholate by rat ileum and jejunum. *Am. J. Physiol.* **259**, G233-G238.

56 Cistola, D.P., Sacchettini, J.C., Banaszak, L.J., Walsh, M.T. and Gordon, J.I. (1989) Fatty acid interactions with rat intestinal and liver fatty acid-binding proteins expressed in *Escherichia coli*. A comparative 13C NMR study. *J. Biol. Chem.* **264**, 2700-2710.

57 Bell, R.M., Ballas, L.M. and Coleman, R.A. (1981) Lipid topogenesis. *J. Lipid Res.* **22**, 391-403.

58 Morre, D.J. (1977) Golgi apparatus and membrane biogenesis. *Cell. Surf. Rev.* **4**, 1-83.

59 Sabesin, S.M. and Frase, S. (1977) Electron microscopic studies of the assembly, intracellular transport, and secretion of chylomicrons by rat intestine. *J. Lipid Res.* **18**, 496-511.

60 Porter, C.J.H. and Charman, W.N. (1997) Uptake of drugs into the intestinal lymphatics after oral administration. *Adv. Drug Deliv. Rev.* **25**, 71-89.

61 Kiyasu, J.Y., Bloom, B. and Chaikoff, I.L. (1952) The portal transport of absorbed fatty acids. *J. Biol. Chem.* **199**, 415-419.

62 Cortot, A., Phillips, S.F. and Malagelada, J.R. (1982) Parallel gastric emptying of nonhydrolyzable fat and water after a solid-liquid meal in humans. *Gastroenterology* **82**, 877-881.

63 Schwizer, W., Asal, K., Kreiss, C., Mettraux, C., Borovicka, J., Remy, B., Guzelhan, C., Hartmann, D. and Fried, M. (1997) Role of lipase in the regulation of upper gastrointestinal function in humans. *Am. J. Physiol.* **273**, G612-G620.

64 Cortot, A., Phillips, S.F. and Malagelada, J.R. (1978) Different rates of fat absorption from homogenised and solid meals (Abstract). *Gut* **19**, A968.

65 Bates, T.R. and Sequeira, J.A. (1975) Bioavailability of micronized griseofulvin from corn oil-in-water emulsion, aqueous suspension, and commercial tablet dosage forms in humans. *J. Pharm. Sci.* **64**, 793-797.

66 Carrigan, P.J. and Bates, T.R. (1973) Biopharmaceutics of drugs administered in lipid-containing dosage forms I: GI absorption of griseofulvin from an oil-in-water emulsion in the rat. *J. Pharm. Sci.* **62**, 1476-1479.

67 Bates, T.R. and Carrigan, P.J. (1975) Apparent absorption kinetics of micronized griseofulvin after its oral administration on single- and multiple-dose regimens to rats as a corn oil-in-water emulsion and aqueous suspension. *J. Pharm. Sci.* **64**, 1475-1481.

68 Chakrabarti, S. and Belpaire, F.M. (1978) Bioavailability of phenytoin in lipid containing dosage forms in rats. *J. Pharm. Pharmac.* **30**, 330-331.

69 Shinkuma, D., Hamaguchi, T., Yamanaka, Y., Mizuno, N. and Yata, N. (1985) Influence of vehicle on gastrointestinal absorption of phenytoin in rats. *Chem. Pharm. Bull.* **33**, 4981-4988.

70 Vetter, R.D., Carey, M.C. and Patton, J.S. (1985) Coassimilation of dietary fat and benzo(a)pyrene in the small intestine: an absorption model using the killifish. *J. Lipid Res.* **26**, 428-434.

71 Muranishi, S., Kimura, T., Ogata, H., Yana, A., Inui, K., Sezaki, H. and Kakemi, K. (1971) In: K. Kakemi (Ed.), Absorption, Metabolism and Excretion of Drugs. Hirokawa, Tokyo, pp. 11.

72 Palin, K.J. and Wilson, C.G. (1984) The effect of different oils on the absorption of probucol in the rat. *J. Pharm. Pharmacol.* **36**, 641-643.

73 Yamahira, Y., Noguchi, T., Takenaka, H. and Maeda, T. (1979) Biopharmaceutical studies of lipid containing oral dosage forms: relationship between drug absorption rate and digestibility of vehicles. *Int. J. Pharm.* **3**, 23-31.

74 Myers, R.A. and Stella, V.J. (1992) Systemic bioavailability of penclomedine (NSC-338720) from oil-in-water emulsions administered intraduodenally to rats. *Int. J. Pharm.* **78**, 217-226.

75 Muranishi, S. (1985) Modification of intestinal absorption of drugs by lipoidal adjuvants. *Pharm. Res.* **2**, 108-118.

76 Fabra-Campos, S., Climent, E., Sanchis-Cortés, A. and Plá-Delfina, J.M. (1994) Compared effects of synthetic and natural bile acid surfactants on xenobiotic absorption: IV. Studies with taurocholate in the rat small intestine. *Int. J. Pharm.* **109**, 197-207.

77 Kararli, T.T. (1989) Gastrointestinal absorption of drugs. In: Critical reviews in therapeutic drug carrier systems. Vol. 6, Issue 1, pp. 39-86.

78 Narawane, L. and Lee, V.H.L. (1994) Absorption barriers. In: A.G. de Boer (Ed.), Drug Absorption Enhancement. Harwood Academic, Switzerland, pp.1-66.

79 Poelma, F.G.J., Breäs, R., Tukker, J.J. and Crommelin, D.J.A. (1991) Intestinal absorption of drugs. The influence of mixed micelles on the disappearance kinetics of drugs from the small intestine of the rat. *J. Pharm. Pharmacol.* **43**, 317-324.

80 DeLong, L.J. and Wylie Nichols, J. (1996) Time-resolved fluorescence anisotropy of fluorescent-labelled lysophospholipid and taurodeoxycholate aggregates. *Biophys. Jr.* **70**, 1466-1471.

81 Grisafe, J.A. and Hayton, W.L. (1978) Effects of short and medium chain fatty acids on absorption of lipophilic drugs from perfused rat intestine. *J. Pharm. Sci.* **67**, 1211-1215.

82 Naylor, L.J., Bakatselou, V., Rodríguez-Hornedo, N., Weiner, N.D. and Dressman, J.B. (1995) Dissolution of steroids in bile salt solutions is modified by the presence of lecithin. *Eur. J. Pharm. Biopharm.* **41**, 346-353.

83 del Estal, J.L., Alvarez, A.I., Villaverde, C. and Prieto, J.G. (1993) Comparative effects of anionic, natural bile acid surfactants and mixed micelles on the intestinal absorption of the anthelmintic albendazole. *Int. J. Pharm.* **91**, 105-109.

84 Kvietys, P.R., Specian, R.D., Grisham, M.B. and Tso, P. (1991) Jejunal mucosal injury and restitution: role of hydrolytic products of food digestion. *Am. J. Physiol.* **261**, G384-G391.

85 Curatolo, W. and Ochoa, R. (1994) Safety assessment of intestinal permeability enhancers. In: A.G. de Boer (Ed.), Drug Absorption Enhancement. Harwood Academic, Switzerland, pp.367-389.

86 Muranishi, S. and Yamamoto, A. (1994) Mechanisms of absorption enhancement through gastrointestinal epithelium. In: A.G. de Boer (Ed.), Drug Absorption Enhancement. Harwood Academic, Switzerland, pp.67-100.

87 Hellman, L., Frazell, E.L. and Rosenfeld, R.S. (1960) Direct measurement of cholesterol absorption via the thoracic duct in man. *J. Clin. Invest.* **39**, 1288-1294.

88 Kuksis, A. (1987) Absorption of fat soluble vitamins. In: A Kuksis (Ed.), Fat Absorption, Vol. 2. CRC Press, Boca Raton, pp.65-86.

89 Caselli, C., Carlier, H. and Bézard, J. (1979) Size of lipoprotein particles in the intestinal lymph of rats fed on corn oil, peanut oil, rapeseed oil or canbra oil. *Nutr. Metab.* **23**, 73-87.

90 Charman, W.N.A. and Stella, V.J. (1986) Effects of lipid class and lipid vehicle volume on the intestinal lymphatic transport of DDT. *Int. J. Pharm.* **33**, 165-172.

91 Sieber, S.M. (1976) The lymphatic absorption of *p,p'*-DDT and some structurally-related compounds in the rat. *Pharmacology* **14**, 443-454.

92 Palin, K.J., Wilson, C.G., Davis, S.S. and Phillips, A.J. (1982) The effect of oils on the lymphatic absorption of DDT. *J. Pharm. Pharmacol.* **34**, 707-710.

93 Nankervis, R., Davis, S.S., Day, N.H. and Shaw, P.N. (1995) Effect of lipid vehicle on the intestinal lymphatic transport of isotretinoin in the rat. *Int. J. Pharm.* **119**, 173-181.

94 Ichihashi, T., Kinoshita, H., Takagishi, Y. and Yamada, H. (1992) Effect of oily vehicles on absorption of mepitiostane by the lymphatic system in rats. *J. Pharm. Pharmacol.* **44**, 560-564.

95 Palin, K.J. (1985) Lipids and oral drug delivery. *Pharmacy International* **November**, 272-275.

96 Charman, W.N. (1992) Lipid vehicle and formulation effects on intestinal lymphatic drug transport. In: W.N. Charman and V.J. Stella (Eds.), Lymphatic Transport of Drugs. CRC Press, Boca Raton, pp.113-179.

97 Charman, W.N. and Stella, V.J. (1986) Estimating the maximal potential for intestinal lymphatic transport of lipophilic drug molecules. *Int. J. Pharm.* **34**, 175-178.

98 Porter, C.J.H., Charman, S.A. and Charman, W.N. (1996) Lymphatic transport of halofantrine in the triple-cannulated anesthetized rat model: Effect of lipid vehicle dispersion. *J. Pharm. Sci.* **85**, 351-356.

99 Porter, C.J.H., Charman, S.A., Humberstone, A.J. and Charman, W.N. (1996) Lymphatic transport of halofantrine in the conscious rat when administered as either the free base or the hydrochloride salt: Effect of lipid class and lipid vehicle dispersion. *J. Pharm. Sci.* **85**, 357-361.

100 Ichihashi, T., Kinoshita, H., Takagishi, Y. and Yamada, H. (1992) Effect of bile on absorption of mepitiostane by the lymphatic system in rats. *J. Pharm. Pharmacol.* **44**, 566-569.

101 Yamahira, Y., Noguchi, T., Takenaka, H. and Maeda,T. (1978) Biopharmaceutical studies of lipid-containing oral dosage forms: Relationship between drug absorption and gastric emptying of lipid formulations. *J. Pharm. Dyn.* **1**, 160-167.

102 Bates, T.R., Pieniaszek, H.J., Sequeira, J.A.L. and Rasmussen, J.E. (1977) Gastrointestinal absorption of griseofulvin from corn oil-in-water emulsions. *Arch. Dermatol.* **113**, 302-306.

103 Charman, W.N., Rogge, M.C., Boddy, A.W. and Berger, B.M. (1993) Effect of food and a monoglyceride emulsion formulation on danazol bioavailability. *J. Clin. Pharmacol.* **33**, 381-386.

104 Gibaldi, M. and Feldman, S. (1970) Mechanisms of surfactant effects on drug absorption. *J. Pharm. Sci.* **59**, 579-589.

105 Macek, K.J. and Krzeminski, S.F. (1975) Susceptibility of bluegill sunfish (Lepomis macrochirus) to non-ionic surfactants. *Bull. Environ. Contam. Toxicol.* **13**, 377-384.

106 Smyth, H.F. and Calandra, J.C. (1969) Toxicologic studies of alkylphenol polyoxyethylene surfactants. *Tox. Appl. Pharmacol.* **14**, 315-334.

107 Moore, R., Carlson, S. and Madara, J.L. (1989) Rapid barrier restitution in an *in vitro* model of intestinal epithelial injury. *Lab. Invest.* **60**, 237-244.

108 MacGregor, K.J., Embleton, J.K., Lacy, J.E., Perry, E.A., Solomon, L.J., Seager, H. and Pouton, C.W. (1997) Influence of lipolysis on drug absorption from the gastro-intestinal tract. *Adv. Drug Delivery Rev.* **25**, 33-46.

109 Dixon, M. and Webb, E.C. (1979) Enzymes, 3rd edition. Academic Press Inc., New York.

110 Burnett, J. (1996) Comparative pharmacokinetics of fenofibrate in dogs using three capsule formulations of fenofibrate. Report No. 530/22-1010, Corning Hazleton (Report for R P Scherer Ltd.), Harrogate.

111 Burnett, J. (1996) Comparative pharmacokinetics of cinnarizine in dogs using three formulations of cinnarizine. Report No. 530/21-1010, Corning Hazleton (Report for R P Scherer Ltd.), Harrogate.

112 Oh, D., Curl, R.L. and Amidon, G.L. (1993) Estimating the fraction dose absorbed from suspensions of poorly soluble compounds in humans: A mathematical model. *Pharm. Res.* **10**, 264-270.

113 Brocklehurst, K. (1992) Electrochemical assays: the pH-stat. In: R. Eisenthal and M.J. Danson (Eds.), Enzyme Assays: A practical approach. IRL Press, Oxford, pp.191-216.

114 Borgström, B. and Erlanson, C. (1973) Pancreatic lipase and co-lipase. Interactions and effects of bile salts and other detergents. *Eur. J. Biochem.* **37**, 60-68.

115 Ziomek, E., Douchet, I., Ivanova, M. and Verger, R. (1996) Interaction of a poly(dimethylsiloxane) with triglycerides in monomolecular films and application to lipase kinetics. *Chem. Phys. Lipids.* **81**, 1-9.

116 Challis, D. (1991) Physicochemical and biopharmaceutical studies of novel self-emulsifying systems for administration by the oral route (SEDDS). Ph.D. thesis, University of Bath.

117 Segel, I.H. (1975) Enzyme kinetics. Behavior and analysis of rapid equilibrium and steady-state enzyme systems. Wiley-Interscience Inc., Toronto.

118 Bowman, W.C., Rand, M.J. and West, G.B. (1967) Textbook of Pharmacology. Blackwell, London.

119 Gurr, M.I. and James, A.T. (1971) Lipid Biochemistry: an introduction. Chapman and Hall Ltd., London.

120 Breg, J.N., Sarda, L., Cozzone, P.J., Rugani, N., Boelens, R. and Kaptein, R. (1995) Solution structure of porcine pancreatic procolipase as determined from H-1 homonuclear 2-dimensional and 3-dimensional NMR. *Eur. J. Biochem.* **227**, 663-672.

121 Vandermeers, A., Vandermeers-Piret, M.C., Rathé, J. and Christophe, J. (1974) On human pancreatic triacylglycerol lipase: isolation and some properties. *Biochim. Biophys. Acta.* **370**, 257-268.

122 U.S. Pharmacopeia National Formulary (1990) USP-XXII, NF-XVII, 22nd revision. United States Pharmacopeial Convention Inc., Rockville.

123 British Pharmacopoeia 1968, The General Medical Council. The Pharmaceutical Press, London.

124 Martindale: The Extra Pharmacopoeia, 28th revision. (1982) J.E.F. Reynolds (Ed.), The Pharmaceutical Press, London.

125 Patton, J.S. (1981) Gastrointestinal lipid digestion. In: L.R. Johnson (Ed.), Physiology of the gastrointestinal tract. Raven Press, New York, pp.1129-1132.

126 Mattson, F.H. and Volpenhein, R.A. (1963) The specific distribution of unsaturated fatty acids in the triglycerides of plants. *J. Lipid Res.* **4**, 392-396.

127 Hüls product information. Hüls AG, D-W-4370, Marl, Postf. 1320.

128 Borgström, B. (1954) Effect of tauro-cholic acid on the pH/activity curve of rat pancreatic lipase. *Biochim. Biophys. Acta.* **13**, 149-150.

129 Borgström, B. and Donnér, J. (1977) The polar interactions between pancreatic lipase, colipase and the triglyceride substrate. *FEBS Lett.* **83**, 23-26.

130 Russell, T.L., Berardi, R.R., Barnett, J.L., Dermentzoglou, L.C., Jarvenpaa, K.M., Schmaltz, S.P. and Dressman, J.B. (1993) Upper gastrointestinal pH in seventy-nine healthy, elderly, North American men and women. *Pharm. Res.* **10**, 187-196.

131 Verger, R. (1984) Pancreatic lipase. In: B. Borgström and H.L. Brockman (Eds.), Lipases. Elsevier, Amsterdam, pp. 83-150.

132 Tipton, K.F. (1992) Principles of enzyme assay and kinetic studies. In: R. Eisenthal and M.J. Danson (Eds.), Enzyme Assays: A practical approach. IRL Press, Oxford, pp.1-58.

133 Segel, I.H. (1975) Enzyme kinetics. Behavior and analysis of rapid equilibrium and steady-state enzyme systems. Wiley-Interscience Inc., Toronto, pp.77-78.

134 Benzonana, G. and Desnuelle, P. (1968) Action of some effectors on the hydrolysis of long-chain triglycerides by pancreatic lipase. *Biochim. Biophys. Acta.* **164**, 47-58.

135 Michaelis, L. and Menten, M.L. (1913) Die kinetik der invertinwirkung. *Biochem. Z.* **49**, 333-369.

136 Brockman, H.L. (1984) General features of lipolysis: reaction scheme, interfacial structure and experimental approaches. In: B. Borgström and H.L. Brockman (Eds.), Lipases. Elsevier, Amsterdam, pp.1-46.

137 Brockman, H.L., Law, J.H. and Kézdy, F.J. (1973) Catalysis by adsorbed enzymes. *J. Biol. Chem.* **248**, 4965-4970.

138 Miller, K.W. and Small, D.M. (1980) Core and surface composition of triglyceride-rich lipoproteins and remnants. *Circulation* **62**, Supplement III, 45.

139 Wieloch, T., Borgström, B., Piéroni, G., Pattus, F. and Verger, R. (1982) Product activation of pancreatic lipase: Lipolytic enzymes as probes for lipid/water interfaces. *J. Biol. Chem.* **257**, 11523-11528.

140 Lindstrom, M., Ljusberg-Wahren, H., Larsson, K. and Borgström, B. (1981) Aqueous lipid phases of relevance to intestinal fat digestion and absorption. *Lipids* **16**, 749-754.

141 Momsen, W.E., Smaby, J.M. and Brockman, H.L. (1979) Interfacial structure and lipase action. Characterization of taurodeoxycholate-didecanoyl-glycerol monolayers by physical and kinetic methods. *J. Biol. Chem.* **254**, 8855-8860.

142 Linthorst, J.M., Clark, S.B. and Holt, P.R. (1977) Triglyceride emulsification by amphipaths present in the intestinal lumen during fat digestion. *J. Colloid Interface Sci.* **60**, 1-10.

143 Benzonana, G. and Desnuelle, P. (1965) Etude cinétique de l'action de la lipase pancrétique sur des triglycerides en émulsion. Essai d'une enzymologie en milieu heterogène. *Biochim. Biophys. Acta* **105**, 121-136.

144 BASF Technical Leaflet, Register 5, MEF 069 e (1988).

145 Henderson, P.J.F. (1992) Statistical analysis of enzyme kinetic data. In: R. Eisenthal and M.J. Danson (Eds.), Enzyme Assays: A practical approach. IRL Press, Oxford, pp.277-316.

146 Eadie, G.S. (1952) On the evaluation of the constants V_m and K_m in enzyme reactions. *Science* **116**, 688.

147 Hofstee, B.H.J. (1952) On the evaluation of the constants V_m and K_m in enzyme reactions. *Science* **116**, 329-331.

148 Dowd, J.E. and Riggs, D.S. (1965) A comparison of estimates of Michaelis-Menten kinetic constants from various linear transformations. *J. Biol. Chem.* **240**, 863-869.

149 Johansen, G. and Lumry, R. (1961) Statistical analysis of enzymic steady-state rate data. *Compt. rend. trav. lab. Carlsberg* **32**, 185-214.

150 Eisenthal, R. and Cornish-Bowden, A. (1974) The direct linear plot. A new graphical procedure for estimating enzyme kinetic parameters. *Biochem. J.* **139**, 715-720.

151 Cornish-Bowden, A. (1979) Fundamentals of Enzyme Kinetics. Butterworths, London.

152 Cornish-Bowden, A. and Eisenthal, R. (1974) Statistical considerations in the estimation of enzyme kinetic parameters by the direct linear plot and other methods. *Biochem. J.* **139**, 721-730.

153 Enzpack, Version 3. Copyright P.A. Williams (1989) Published and distributed by Biosoft, 22 Hills Rd, Cambridge.

154 Microcal Origin, Version 2.94. Copyright MicroCal Software Inc. (1991-1993) USA.

155 Cleland, W.W. (1967) The statistical analysis of enzyme kinetic data. *Adv. Enzymol.* **29**, 1-32.

156 Gargouri, Y., Ransac, S. and Verger, R. (1997) Covalent inhibition of digestive lipases: An *in vitro* study. *Biochim. Biophys. Acta.* **1344**, 6-37.

157 Lineweaver, H. and Burk, D. (1934) The determination of enzyme dissociation constants. *J. Amer. Chem. Soc.* **56**, 658-666.

158 Morris, J.G. (1974) A Biologist's Physical Chemistry, 2nd edition. Edward Arnold Ltd., London.

159 Hermoso, J., Pignol, D., Kerfelec, B., Crenon, I., Chapus, C. and Fontecilla-Camps, J.C. (1996) Lipase activation by non-ionic detergents: The crystal structure of the porcine lipase-colipase-tetraethylene glycol monooctyl ether complex. *J. Bio. Chem.* **271**, 18007-18016.

160 Verger, R., Mieras, M.C.E. and de Haas, G.H. (1973) Action of phospholipase A at interfaces. *J. Bio. Chem.* **248**, 4023-4034.

161 Hauser, H., Guyer, W. and Howell, K. (1979) Lateral distribution of negatively charged lipids in lecithin membranes. Clustering of fatty acids. *Biochemistry* **18**, 3285-3291.

162 Neuman, R.D. (1975) Calcium binding in stearic acid monomolecular films. *J. Colloid Interface Sci.* **53**, 161-171.

163 Borgström, B. (1980) The importance of phospholipids, pancreatic phospholipase A_2, fatty acid and other factors for the digestion of dietary fat: *in vitro* experiments with porcine enzymes. *Gastroenterology* **78**, 954-962.

164 Rosenthal, A.F. and Pousada, M. (1968) Inhibition of phospholipase C by phosphonate analogs of glycerophosphatides. *Biochim. Biophys. Acta* **164**, 226-237.

165 Florence, A.T. and Attwood, D. (1986) Physicochemical principles of pharmacy. Macmillan Education Ltd., Basingstoke.

166 Hartley, G.S. (1936) Aqueous solutions of paraffin chain salts. Hermann and Cie, Paris.

167 Griffin, W.C. (1949) Classification of surface active agents by HLB. *J. Soc. Cosmet. Chem.* **1**, 311-326.

168 Griffin, W.C. (1949) Calculation of HLB values of non-ionic surfactants. *J. Soc. Cosmet. Chem.* **5**, 249-256.

169 Shinoda, K. and Arai, H. (1964) The correlation between phase inversion temperature in emulsion and cloud point in solution of nonionic emulsifier. *J. Phys. Chem.* **68**, 3485-3490.

170 Marszall, L. (1987) HLB of nonionic surfactants: PIT and EIP methods. In: M.J. Schick (Ed.), Nonionic surfactants: Physical chemistry volume 23. Marcel Dekker Inc., New York, pp.493-547.

171 Shinoda, K. and Friberg, S. (1986) Emulsions and solubilization. John Wiley and Sons Inc., New York.

172 Becher, P. and Schick, M.J. (1987) Macroemulsions. In: M.J. Schick (Ed.), Nonionic surfactants: Physical chemistry volume 23. Marcel Dekker Inc., New York, pp.435-491.

173 Borgström, B. (1976) Binding of pancreatic colipase to interfaces: effects of detergents. *FEBS Lett.* **71**, 201-204.

174 Borgström, B. (1977) Physico-chemical characteristics of the lipase-colipase-bile salt system. In: P. Boulanger, M.F. Jayle and J. Roche (Eds.), Exposés Annuels de Biochimie Médicale. Masson, Paris, pp.173-182.

175 Piéroni, G., Gargouri, Y., Sarda, L. and Verger, R. (1990) Interactions of lipases with lipid monolayers - facts and questions. *Adv. Colloid Interface Sci.* **32**, 341-378.

176 Verger, R., Rietsch, J. and Desnuelle, P. (1977) Effects of colipase on hydrolysis of monomolecular films by lipase. *J. Biol. Chem.* **252**, 4319-4325.

177 Borgström, B. (1977) The action of bile salts and other detergents on pancreatic lipase and the interaction with colipase. *Biochim. Biophys. Acta* **488**, 381-391.

178 Borgström, B., Wieloch, T. and Erlanson-Albertsson, C. (1979) Evidence for a pancreatic procolipase and its activation by trypsin. *FEBS Lett.* **108**, 407-410.

179 Piéroni, G. and Verger, R. (1979) Hydrolysis of mixed monomolecular films of triglyceride/lecithin by pancreatic lipase. *J.Biol. Chem.* **254**, 10090-10094.

180 Hollis, G.L. (1982) Surfactants Europa volume 1. George Godwin, Harlow.

181 The Merck Index 12th edition. (1996) S. Budavari, (Ed.), Merck & Co. Inc., New Jersey.

182 Gunstone, F.D., Harwood, J.L. and Padley, F.B. (1994) The Lipid Handbook 2nd edition. Chapman and Hall, London.

183 Crills and Crillets data sheet. Croda Surfactants Ltd., Goole, North Humberside.

184 Gattefossé product information. Gattefossé corporation, Westchester Plaza, Elmsford, New York, 10523.

185 Schmitt, T.M. (1992) Analysis of surfactants volume 40. Marcel Dekker Inc., New York.

186 Williams, D.H. and Fleming, I. (1989) Spectrocopic methods in organic chemistry 4th edition. McGraw-Hill (UK) Ltd., Maidenhead.

187 Ribeiro, A.A. and Dennis, E.A. (1987) Structure and dynamics by NMR and other methods. In: M.J. Schick (Ed.), Nonionic surfactants: Physical chemistry volume 23. Marcel Dekker Inc., New York, pp.971-1009.

188 Pouton, C.W. (1997) Formulation of self-emulsifying drug delivery systems. *Adv. Drug Delivery Rev.* **25**, 47-58.

189 Groves, M.J. and Mustafa, R.M.A. (1974) Measurement of the 'spontaneity' of self-emulsifiable oils. *J. Pharm. Pharmacol.* **26**, 671-681.

190 Wakerly, M.G., Pouton, C.W., Meakin, B.J. and Morton, F.S. (1986) Self-emulsification of vegetable oil-nonionic surfactant mixture: a proposed mechanism of action. *Am. Chem. Soc. Symposium Series* **311**, 242-255.

191 Shah, N.H., Carvajal, M.T., Patel, C.I., Infeld, M.H. and Malick, A.W. (1994) Self-emulsifying drug delivery systems with polyglycolyzed glycerides for improving *in vitro* dissolution and oral absorption of lipophilic drugs. *Int. J. Pharm.* **106**, 15-23.

192 Bachynsky, M.O., Shah, N.H., Patel, C.I. and Malick, A.W. (1997) Factors affecting the efficiency of a self-emulsifying oral delivery system. *Drug Dev. Ind. Pharm.* **23**, 809-816.

193 Constantinides, P.P. (1995) Lipid microemulsions for improving drug dissolution and oral absorption: physical and biopharmaceutical aspects. *Pharm. Res.* **12**, 1561-1572.

194 Pouton, C.W. (1985) Effects of the inclusion of a model drug on the performance of self-emulsifying formulations. *J. Pharm. Pharmac.* **37**, 1P.

195 Lawrence, M.J. (1996) Microemulsions as drug delivery vehicles. *Current Opinion in Coll. & Inter. Sci.* **1**, 826-832.

196 Ptachcinski, R.J., Venkataramanan, R. and Burckart, G.J. (1986) Clinical pharmacokinetics of cyclosporin. *Clin. Pharmacokinet.* **11**, 107-132.

197 Drewe, J., Beglinger, C. and Kissel, T. (1992) The absorption site of cyclosporin in the human gastrointestinal tract. *Br. J. Clin. Pharmac.* **33**, 39-43.

198 Tarr, B.D. and Yalkowsky, S.H. (1989) Enhanced intestinal absorption of cyclosporine in rats through the reduction of emulsion droplet size. *Pharm. Res.* **6**, 40-43.

199 Noble, S. and Markham, A. (1995) Cyclosporin: A review of the pharmacokinetic properties, clinical efficacy and tolerability of a microemulsion-based formulation (Neoral®). *Drugs* **50**, 924-941.

200 Mueller, E.A., Kovarik, J.M., van Bree, J.B., Grevel, J., Lücker, P.W. and Kutz, K. (1994) Influence of a fat-rich meal on the pharmacokinetics of a new oral formulation of cyclosporine in a crossover comparison with the market formulation. *Pharm. Res.* **11**, 151-155.

201 Trull, A.K., Tan, K.K.C., Tan, L., Alexander, G.J.M. and Jamieson, N.V. (1995) Absorption of cyclosporin from conventional and new microemulsion oral formulations in liver transplant recipients with external biliary diversion. *Br. J. Clin. Pharmac.* **39**, 627-631.

202 Kovarik, J.M., Mueller, E.A., van Bree, J.B., Tetzloff, W. and Kutz, K. (1994) Reduced inter- and intraindividual variability in cyclosporine pharmacokinetics from a microemulsion formulation. *J. Pharm. Sci.* **83**, 444-446.

203 Mueller, E.A., Kovarik, J.M., van Bree, J.B., Tetzloff, W., Grevel, J. and Kutz, K. (1994) Improved dose linearity of cyclosporine pharmacokinetics from a microemulsion formulation. *Pharm. Res.* **11**, 301-304.

204 Charman, S.A., Charman, W.N., Rogge, M.C., Wilson, T.D., Dutko, F.J. and Pouton, C.W. (1992) Self-emulsifying drug delivery systems: formulation and biopharmaceutical evaluation of an investigational lipophilic compound. *Pharm. Res.* **9**, 87-93.

205 Lin, J.H., Chen, I. and Lievens, H. (1991) The effect of dosage forms on the oral absorption of L-365 260, a potent CCK_B receptor antagonist in dogs. *Pharm. Res.* **8**, S-272 (Abstract).

206 Tokumura, T., Tsushima, Y., Tatsuishi, K., Kayano, M., Machida, Y. and Nagai, T. (1987) Enhancement of the oral bioavailability of cinnarizine in oleic acid in beagle dogs. *J. Pharm. Sci.* **76**, 286-288.

207 Corning Hazleton (Europe), Otley Rd., Harrogate, North Yorkshire HG3 1PY.

208 Burnett, J. and Thompson, T. (1994) Comparative pharmacokinetics of progesterone in dogs using three capsule formulations of progesterone. Report No. 530/20-1010, Corning Hazleton (Report for R P Scherer Ltd.), Harrogate.

209 Saunders, L. (1971) Principles of Physical Chemistry for Biology and Pharmacy. Oxford University Press, London.

210 Bates, T.R., Gibaldi, M. and Kanig, J.L. (1966) Solubilizing properties of bile salt solutions. II Effect of inorganic electrolyte, lipids, and a mixed bile salt system on solubilization of glutethimide, griseofulvin, and hexestrol. *J. Pharm. Sci.* **55**, 901-906.

211 Mcbain, M.E.L. and Hutchinson, E. (1955) Solubilization and related phenomena. Academic Press Inc., New York.

212 Bates, T.R., Gibaldi, M. and Kanig, J.L. (1966) Rate of dissolution of griseofulvin and hexoestrol in bile salt solutions. *Nature* **5043**, 1331-1333.

213 Bates, T.R., Gibaldi, M. and Kanig, J.L. (1966) Solubilizing properties of bile salt solutions I Effect of temperature and bile salt concentration on solubilization of glutethimide, griseofulvin, and hexestrol. *J. Pharm. Sci.* **55**, 191-199.

214 Meli, A., Cargill, D.I., Giannina, T. and Steinetz, B.G. (1968) Studies on the transport of estrogens by the rat small intestine *in vivo*. *Proc. Soc. Exp. Biol. Med.* **129**, 937-944.

215 Nightingale, C.H., Axelson, J.E. and Gibaldi, M. (1971) Physiologic surface-active agents and drug absorption. VIII: Effect of bile flow on sulfadiazine absorption in the rat. *J. Pharm. Sci.* **60**, 145-147.

216 Miyazaki, S., Inoue, H., Yamahira, T. and Nadai, T. (1979) Interaction of drugs with bile components. I. Effects of bile salts on the dissolution behavior of indomethacin and phenylbutazone. *Chem. Pharm. Bull.* **27**, 2468-2472.

217 Bakatselou, V., Oppenheim, R.C. and Dressman, J.B. (1991) Solubilization and wetting effects of bile salts on the dissolution of steroids. *Pharm. Res.* **8**, 1461-1469.

218 Bates, T.R., Lin, S. and Gibaldi, M. (1967) Solubilization and rate of dissolution of drugs in the presence of physiologic concentrations of lysolecithin. *J. Pharm. Sci.* **56**, 1492-1495.

219 Small, D.M. (1967) Physicochemical studies of cholesterol gallstone formation. *Gastroenterology* **52**, 607-610.

220 Mazer, N.A., Benedek, G.B. and Carey, M.C. (1980) Quasielastic light-scattering studies of aqueous biliary lipid systems. Mixed micelle formation in bile salt-lecithin solutions. *Biochemistry* **19**, 601-615.

221 Hjelm, R.P., Thiyagarajan, P. and Alkan-Onyuksel, H. (1992) Organization of phosphatidylcholine and bile salt in rodlike mixed micelles. *J. Phys. Chem.* **96**, 8653-8661.

222 Walter, A., Vinson, P.K., Kaplun, A. and Talmon, Y. (1991) Intermediate structures in the cholate-phosphatidylcholine vesicle micelle transition. *Biophys. J.* **60**, 1315-1325.

223 Egelhaaf, S.U. and Schurtenberger, P. (1994) Shape transformations in the lecithin-bile salt system: From cylinders to vesicles. *J. Phys. Chem.* **98**, 8560-8573.

224 Long, M.A., Kaler, E.W., Lee, S.P. and Wignall, G.D. (1994) Characterization of lecithin-taurodeoxycholate mixed micelles using small-angle neutron scattering and static and dynamic light scattering. *J. Phys. Chem.* **98**, 4402-4410.

225 Kozlov, M.M., Lichtenberg, D. and Andelman, D. (1997) Shape of phospholipid/surfactant mixed micelles: Cylinders or disks? Theoretical analysis. *J. Phys. Chem.* B **101**, 6600-6606.

226 Pedersen, J.S., Egelhaaf, S.U. and Schurtenberger, P. (1995) Formation of polymerlike mixed micelles and vesicles in lecithin-bile salt solutions: Small-angle neutron-scattering study. *J. Phys. Chem.* **99**, 1299-1305.

227 Small, D.M., Penkett, S.A. and Chapman, D. (1969) Studies on simple and mixed bile salt micelles by nuclear magnetic resonance spectroscopy. *Biochim. Biophys. Acta.* **176**, 178-189.

228 Uematsu, S., Uchida, T., Kinoshita, A., Kimura, F. and Akahori, Y. (1995) Relation between micellar structure of model bile and activity of esterase. *Biochim. Biophys. Acta.* **1258**, 122-134.

229 Briganti, G., D'Archivio, A.A., Galantini, L. and Giglio, E. (1996) Structural study of the micellar aggregates of sodium and rubidium glyco- and taurodeoxycholate. *Langmuir* **12**, 1180-1187.

230 Bottari, E. and Festa, M.R. (1996) On the composition of sodium taurodeoxycholate micellar solutions. *Langmuir* **12**, 1777-1783.

231 Rautureau, M., Bisalli, A. and Rambaud, J.-C. (1981) Bile salts and lipids in aqueous intraluminal phase during the digestion of a standard meal in normal man. *Gastroenterol. Clin. Biol.* **5**, 417-425.

232 Rosoff, M. and Serajuddin, A.T.M. (1980) Solubilization of diazepam in bile salts and in sodium cholate-lecithin-water phases. *Int. J. Pharm.* **6**, 137-146.

233 Naylor, L.J., Bakatselou, V. and Dressman, J.B. (1993) Comparison of the mechanism of dissolution of hydrocortisone in simple and mixed micelle systems. *Pharm. Res.* **10**, 865-870.

234 Braun, R.D. (1985) Introduction to Chemical Analysis. McGraw-Hill Inc., Singapore.

235 Harris, R.A. and Solomon, L.J. (1996) Solubilization of steroid drugs by bile salt / lecithin mixed micelles (unpublished results). University of Bath, Bath.

236 Thakkar, A.L. (1970) Solubilization of some steroid hormones in aqueous solutions of bile salts. *J. Pharm. Sci.* **59**, 1499-1501.

237 Martis, L., Hall, N.A. and Thakkar, A.L. (1972) Micelle formation and testosterone solubilization by sodium glycocholate. *J. Pharm. Sci.* **61**, 1757-1761.

238 Ekwall, P., Fontell, K. and Sten, A. (1957) Proceedings 2nd International Congress on Surface Activity. I Gas / liquid and liquid / liquid interface. Butterworths, London. pp.357-373.

239 Mithani, S.D., Bakatselou, V., TenHoor, C.N. and Dressman, J.B. (1996) Estimation of the increase in solubility of drugs as a function of bile salt concentration. *Pharm. Res.* **13**, 163-167.

240 Hörter, D. and Dressman, J.B. (1997) Influence of physicochemical properties on dissolution of drugs in the gastrointestinal tract. *Adv. Drug Deliv. Rev.* **25**, 3-14.

241 Collett, J.H. and Koo, L. (1975) Interaction of substituted benzoic acids with polysorbate 20 micelles. *J. Pharm. Sci.* **64**, 1253-1255.

242 Tomida, H., Yotsuyanagi, T. and Ikeda, K. (1978) Solubilization of steroid hormones by polyoxyethylene lauryl ether. *Chem. Pharm. Bull.* **26**, 2832-2837.

243 Stearns, R.S., Oppenheimer, H., Simon, E. and Harkins, W.D. (1947) Solubilization by solutions of long-chain colloidal electrolytes. *J. Chem. Phys.* **15**, 496-507.

244 Miyazaki, S., Yamahira, T., Morimoto, Y. and Nadai, T. (1981) Micellar interaction of indomethacin and phenylbutazone with bile salts. *Int. J. Pharm.* **8**, 303-310.

245 Elworthy, P.H., Florence, A.T. and Macfarlane, C.B. (1968) Solubilization by surface-active agents and its applications in chemistry and the biological sciences. Chapman and Hall Ltd., London.

246 Sjöblöm, L. (1965) Surface Chemistry. Munkesgaard, Copenhagen.

247 Klevens, H.B. (1950) Solubilization. *Chem. Rev.* **47**, 1-74.

248 Cai, X., Grant, D.J.W. and Wiedmann, T.S. (1997) Analysis of the solubilization of steroids by bile salt micelles. *J. Pharm. Sci.* **86**, 372-377.

249 Scherstén, T. (1973) Formation of lithogenic bile in man. *Digestion* **9**, 540-553.

250 Rekker, R.F. (1977) The Hydrophobic Fragmental Constant. Elsevier Scientific, Amsterdam.

251 Shankland, W. (1970) The equilibrium and structure of lecithin-cholate mixed micelles. *Chem. Phys. Lipids* **4**, 109-130.

252 O'Reilly, J.R., Corrigan, O.I. and O'Driscoll, C.M. (1994) The effect of mixed micellar systems, bile salt / fatty acids, on the solubility and intestinal absorption of clofazimine (B663) in the anaesthetised rat. *Int. J. Pharm.* **109**, 147-154.

253 Luner, P.E., Babu, S.R. and Radebaugh, G.W. (1994) The effects of bile salts and lipids on the physicochemical behavior of gemfibrozil. *Pharm. Res.* **11**, 1755-1760.

254 De Smidt, J.H., Grit, M. and Crommelin, D.J.A. (1994) Dissolution kinetics of griseofulvin in mixed micellar solutions. *J. Pharm. Sci.* **83**, 1209-1212.

255 Gupta, S.L., Higuchi, W.I. and Ho, N.F.H. (1985) Cholesterol monohydrate dissolution rate studies in aqueous micellar sodium chenodeoxycholate solutions. *J. Pharm. Sci.* **74**, 1178-1183.

256 Borgström, B. (1985) The micellar hypothesis of fat absorption: Must it be revisited? *Scand. J. Gastroenterol.* **20**, 389-394.

257 Ganong, W.F. (1997) Review of Medical Physiology 18th edition. Appleton & Lange, Connecticut.

258 Fujita, T., Isawa, J. and Hansch, C. (1964) A new substituent constant, π, derived from partition coefficients. *J. Am. Chem. Soc.* **86**, 5175-5180.

259 Leo, A., Hansch, C. and Elkins, D. (1971) Partition coefficients and their uses. *Chem. Rev.* **71**, 525-616.

260 Könemann, H., Zelle, R., Busser, F. and Hammers, W.E. (1979) Determination of log P_{oct} values of chloro-substituted benzenes, toluenes and anilines by high-performance liquid chromatography on ODS-silica. *J. Chromatogr.* **178**, 559-565.

261 Hafkenscheid, T.L. and Tomlinson, E. (1986) Estimation of physicochemical properties of organic solutes using HPLC retention parameters. *Adv. Chromatogr.* **25**, 1-62.

262 Hansch, C. and Leo, A.J. (1979) Substituent Constants for Correlation Analysis in Chemistry and Biology. John Wiley and Sons Inc., New York.

263 Haggerty, W.J. and Murrill, E.A. (1974) Applications of HPLC in contract research. *Res. Devel.* **25**, 30-35.

264 Terada, H. (1986) Determination of log P_{oct} by high-performance liquid chromatography, and its application in the study of quantitative structure-activity relationships. *Quant. Struct.-Act. Relat.* **5**, 81-88.

265 Lambert, W.J. (1993) Modeling oil-water partitioning and membrane permeation using reversed-phase chromatography. *J. Chromatogr.* A **656**, 469-484.

266 Miyake, K. and Terada, H. (1978) Preparation of a column with octanol-like properties for high-performance liquid chromatography. Direct measurement of partition coefficients in an octanol-water system. *J. Chromatogr.* **157**, 386-390.

267 Hulshoff, A. and Perrin, J.H. (1976) A comparison of the determination of partition coefficients of 1,4-benzodiazepines by high-performance liquid chromatography and thin-layer chromatography. *J. Chromatogr.* **129**, 263-276.

268 Miyake, K. and Terada, H. (1982) Determination of partition coefficients of very hydrophobic compounds by high-performance liquid-chromatography on glyceryl-coated controlled-pore glass. *J. Chromatogr.* **240**, 9-20.

269 El Tayar, N., van de Waterbeemd, H. and Testa, B. (1985) Lipophilicity measurements of protonated basic compounds by reversed-phase high-performance liquid-chromatography. 2. Procedure for the determination of a lipophilic index measured by reversed-phase high-performance liquid-chromatography. *J. Chromatogr.* **320**, 305-312.

270 Miyake, K., Mizuno, N. and Terada, H. (1986) Method for determination of partition coefficients by high-performance liquid-chromatography on an octadecylsilane column. Examination of its applicability. *Chem. Pharm. Bull.* **34**, 4787-4796.

271 Schedl, H.P. (1965) Absorption of steroid hormones from the human small intestine. *J. Clin. Endocrinol.* **25**, 1309-1316.

272 Dorsey, J.G. and Khaledi, M.G. (1993) Hydrophobicity estimations by reversed-phase liquid chromatography. Implications for biological partitioning processes. *J. Chromatogr.* A **656**, 485-499.

273 Valko, K., Synder, L.R. and Glajch, J.L. (1993) Retention in reversed-phase liquid-chromatography as a function of mobile-phase composition. *J. Chromatogr.* A **656**, 501-520.

274 Schoenmakers, P.J., Billiet, H.A.H., Tijssen, R. and de Galan, L. (1978) Gradient selection in reversed-phase liquid chromatography. *J. Chromatogr.* **149**, 519-537.

275 Schoenmakers, P.J., Billiet, H.A.H. and de Galan, L. (1979) Influence of organic modifiers on the retention behaviour in reversed-phase liquid chromatography and its consequences for gradient elution. *J. Chromatogr.* **185**, 179-195.

276 Yamagami, C. and Takao, N. (1991) Hydrophobicity parameters determined by reversed-phase liquid chromatography. IV. Shortcoming of log K_W approach for prediction of log P of pyrazines. *Chem. Express* **6**, 113-116.

277 Miyake, K., Mizuno, N. and Terada, H. (1988) Effect of hydrogen bonding on the high-performance liquid chromatographic behaviour of organic compounds. Relationship between capacity factors and partition coefficients. *J. Chromatogr.* **439**, 227-235.

278 Amidon, G.L., Lennernäs, H., Shah, V.P. and Crison, J.R. (1995) A theoretical basis for a biopharmaceutic drug classification: The correlation of *in vitro* drug product dissolution and *in vivo* bioavailability. *Pharm. Res.* **12**, 413-420.

279 Sinko, P.J., Leesman, G.D. and Amidon, G.L. (1991) Predicting fraction dose absorbed in humans using a macroscopic mass balance approach. *Pharm. Res.* **8**, 979-988.

280 Yu, L.X., Lipka, E., Crison, J.R. and Amidon, G.L. (1996) Transport approaches to the biopharmaceutical design of oral drug delivery systems: prediction of intestinal absorption. *Adv. Drug Deliv. Rev.* **19**, 359-376.

281 Ho, N.F.H., Merkle, H.P. and Higuchi, W.I. (1983) Mechanistic and physiologically realistic approach to the biopharmaceutical design of oral drug delivery systems. *Drug Dev. Ind. Pharm.* **9**, 1111-1184.

Appendix A

Derivation of equations for emulsion microscopic mass balance model of drug absorption.

In this appendix a derivation is given of the differential equations resulting from the Microscopic Mass Balance (MMB) model for drug delivery through the intestinal membrane from an emulsion, as described in Chapter 7. The model is analogous to that developed by Oh et al[112] for drug delivery from a suspension, but describes the partitioning of the drug from lipid droplets into solution as opposed to a suspension of solid particles dissolving into solution.

In view of this, detailed attention is given only to those aspects of the emulsion MMB model that differ from the suspension MMB model. The paper published by Oh et al[112] gives details of the other approximations that the two models have in common, and the reader is referred to this for further details.

Differential equations

The model consists of an emulsion flowing down an 'intestinal' tube, of length L and radius R, at a volume flow rate of Q. In exactly the same way as for the suspension MMB model the intestinal tube is divided into infinitesimal 'slices' of width dz and radius R. Applying the 'mass balance approach' implies that for each of these slices

(rate of drug flowing in) - (rate of drug flowing out) -
(rate of mass partitioning from lipid droplet to solution) = 0 (A.1)

As for the suspension MMB model this leads to a differential equation,

$$\frac{d}{dz}(\dot{M}_{oil}) + j_P . S_P . \pi R^2 = 0 \qquad \text{(A.2)}$$

where

$$\dot{M}_{oil} = Q.\left(\frac{N_0}{V_0}\right).\frac{4\pi r^3}{3}.C_{oil} \qquad \text{(A.3)}$$

$$j_P = \frac{D}{r}.\left(\frac{C_{oil}}{P} - C\right) \tag{A.4}$$

$$S_P = \left(\frac{N_0}{V_0}\right).4\pi r^2, \tag{A.5}$$

and z is the distance along the intestinal tube.

The same approximations are used for the diffusion properties of the drug as for the suspension MMB model (pseudo steady state transfer at low Reynolds number). The symbols appearing in these equations are defined below:

$\dot{M}_{oil}$	Mass of drug dissolved in lipid droplets flowing through the slice per unit time.
j_P	Rate at which the drug partitions from the lipid droplet to the intestinal fluid, per unit area of partition surface (see Chapter 7 - this equation replaces the Noyes-Whitney equation used for suspension MMB model).
S_P	Total surface area of lipid droplets in the emulsion per unit volume.
C_{oil}	Concentration of drug dissolved in lipid droplets (this is a function of z).
C	Concentration of drug dissolved in intestinal fluid (this is a function of z).
r	Radius of lipid droplets.
Q	Volume flow rate of intestinal fluid.
N_0/V_0	Number of lipid droplets per unit volume.
D	Diffusivity of the drug.
P	Partition coefficient.

From these quantities and Equations A.2-A.5 a differential equation for the concentration of the drug in lipid droplets can be obtained,

$$\frac{dC_{oil}}{dz} = -\frac{3D\pi R^2}{Qr^2}\left(\frac{C_{oil}}{P} - C\right). \tag{A.6}$$

Reducing this to the dimensionless variables, $z^* = z/L$, $C^*_{oil} = C_{oil}/C^{initial}_{oil}$ and $C^* = C/C^{initial}_{oil}$ and introducing the diffusion number, Df, defined as

$$Df = \frac{3D/r^2}{Q/(\pi R^2 L)}, \tag{A.7}$$

gives the equation

$$\frac{dC^*_{oil}}{dz^*} = -Df.\left(\frac{C^*_{oil}}{P} - C^*\right). \tag{A.8}$$

This is Equation 7.6 in Chapter 7.

Next we turn our attention to the transport of drug across the intestinal membrane. The MMB approach gives us the relation:

(rate of dissolved drug flowing in) - (rate of dissolved drug flowing out)
+ (rate of drug partitioning from lipid droplet to solution)
- (rate of drug absorption across intestinal membrane) = 0 (A.9)

or

$$-\frac{d}{dz}(\dot{M}_{sol.}) + j_P.S_P.\pi R^2 - j_W.(2\pi R) = 0 \tag{A.10}$$

where the new symbols are:

$\dot{M}_{sol}$ Mass of drug dissolved in intestinal fluid flowing through the slice per unit time.

j_W Rate at which the drug is adsorbed across intestinal membrane per unit surface area.

Assuming sink conditions[112] for the adsorption across the intestinal membrane these quantities are given by:

$$\dot{M}_{oil} = Q.C \qquad \text{(A.11)}$$

$$j_W = P_{eff}.C \qquad \text{(A.12)}$$

where P_{eff} is the effective permeability of the compound[112]. Reducing Equation A.10 to dimensionless variables,

$$z^* = z/L,$$

$$C^*_{oil} = C_{oil}/C^{initial}_{oil}$$

and

$$C^* = C\ /C^{initial}_{oil}$$

and introducing the volume number, Vn, defined as

$$Vn = \frac{4\pi r^3}{3}.\frac{N_0}{V_0}, \qquad \text{(A.13)}$$

and the absorption number, An, defined as

$$An = \frac{P_{eff}.\pi RL}{Q}, \qquad \text{(A.14)}$$

gives the equation

$$\frac{dC^*}{dz^*} = Df.Vn.\left(\frac{C^*_{oil}}{P} - C^*\right) - 2.An.C^*. \qquad \text{(A.15)}$$

This is Equation 7.7 given in Chapter 7.

Solution of the differential equations

The next step is the solution of Equations A.8 and A.15 in order to calculate the rate at which the drug is delivered. Substituting A.8 into Equation A.15 gives a second order linear differential equation, with solutions of the form

$$C_{oil}(z^*) = Ae^{-\alpha z^*} + Be^{-bz^*} \qquad \text{(A.16)}$$

where A and B depend on the concentration of drug within the lipid droplets at $z = 0$, and a and b depend on Df, Vn, An and P. Equation A.16 implies that the concentration of drug in the lipid decays along the intestine as a sum of two exponential functions. For any realistic values of the drug and intestinal parameters, only one of these decays is significant (here taken as the $Ae^{-a.z}$ contribution). The other term is negligible (due to small B and large b) and is ignored here.

Applying the boundary conditions that all of the drug is in the lipid droplets upon entry into the intestine, we arrive at the solution

$$C_{oil}(z^*) = \exp\left[-\frac{2.An}{1+Vn.P+\frac{2.An.P}{Df}}.z^*\right]. \qquad \text{(A.17)}$$

We can obtain the concentration in the intestinal fluid, $C(z^*)$, by substituting this expression into Equation A.8.

Fraction of dose delivered

We are particularly interested in the fraction of the total drug delivered across the intestinal membrane, F. This is given by

F = 1 - (mass drug out, in lipid + solution) /
(mass drug in, in lipid + solution) (A.18)

or

$$F = 1 - \frac{4/3\pi r^3 . N_0 . C^*_{oil}(1) + C^*(1).V_0}{4/3\pi r^3 . N_0}. \quad (A.19)$$

A further simplification can be made by noting that for the poorly water-soluble drugs of interest the amount of drug in the intestinal fluid is small (close to zero), and makes a negligible contribution to Equation A.19. In view of this, and using Equation A.17, Equation A.19 can be written as

$$F = 1 - \exp\left[- \frac{2.An}{1 + Vn.P + \frac{2.An.P}{Df}} \right]. \quad (A.20)$$

This is Equation 7.9 in Chapter 7.

A further simplification can be made for the case of small lipid droplets. As r becomes smaller, the diffusion number becomes larger, so the fraction $2AnP/Df$ in Equation A.20 becomes negligible. Hence we can say that in the limit $r \rightarrow 0$ the fractional dose absorbed is given by

$$F = 1 - \exp\left[- \frac{2.An}{1 + Vn.P} \right]. \quad (A.21)$$

This gives the fractional dose delivered when lipid droplet size is not a limiting factor in drug delivery, as discussed in Chapter 7.